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Characterization of the Stroma and Epithelial Pathways in the Mammary Gland causing Susceptibility to Cancer

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Characterization of the Stromal and Epithelial Pathways in the Mammary Gland causing Susceptibility to Cancer

By

Alvin Tu Lo

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Comparative Biochemistry

in the

Graduate Division

of the

University of California, Berkeley

Committee in Charge:

Professor Mina J. Bissell, Co-Chair
Professor Fenyong Liu, Co-Chair
Professor Sangwei Lu
Professor Danica Chen

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Abstract

Characterization of the Stromal and Epithelial Pathways in the Mammary Gland causing Susceptibility to Cancer

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Doctor of Philosophy in Comparative Biochemistry

University of California, Berkeley

Professor Mina J. Bissell, Co-Chair

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It is now known, the microenvironment including the stroma play an important role in both organ specificity and mammary cancer. In characterizing the interactions between stroma and epithelium, it is useful to develop an *ex vivo* model to more freely dissect out the intricate network of signals that are necessary to allow functional differentiation *in vivo*. With this in mind, my first aim was to develop such models to study the mammary gland outside the animal in designer microenvironments. It has been known for some time that once cells are removed from their native tissue environment and placed into traditional two-dimensional (2D) cultures, cells lose functional performance and relevant morphology (M.J. Bissell 1981). In 1977, Emerman and Pitelka using a technique developed by Michalopoulas and Pitot placed mammary cells on top of collagen-1 gels and allowed it to float (Michalopoulos & Pitot 1975; Emerman & Pitelka 1977). In the presence of lactogenic hormones, mammary cells were able to produce milk proteins. These studies were reproduced and colleagues showed that in the floating collagen gel the important component produced by the cells was laminin-111 (Danielson et al. 1984; Parry et al. 1987). Following these studies, the Bissell laboratory discovered in 1989 that a gel mimicking the properties of the basement membrane, a specialized form of extracellular matrix in glandular tissues, allows mammary cells to produce milk and secrete it vectorially (Barcellos-Hoff et al. 1989; Streuli et al. 1991; Streuli et al. 1995). In this dissertation, I utilized an organoid technique developed in the Bissell laboratory to recapitulate both form and function of mammary gland from small pieces of mammary tissue. By using these culture models, we were able to systematically define the biochemical and environmental signaling cues that are important in mammary gland form and function. As such, we have composed and detailed a number of matrices to reproduce the developmental processes *ex vivo* similar to what is observed *in vivo* in the developing mammary gland (Lo et al. 2012). These methodologies illustrate a way to investigate elaborately the epithelium outside the complex microenvironment of the tissue, and provide a system for investigating not only normal developmental processes but also diseases such as cancer. We then applied the use of these three-dimensional

(3D) culture models to investigate the developmental processes of mammary gland branching.

Invasion is a key step of branching morphogenesis, the process by which simple epithelial structures form elaborate branched networks (Williams & Daniel 1983; Montesano et al. 1991; Hirai et al. 1998; Simian et al. 2001; Fata et al. 2007). This process requires invasion through a type-I collagen rich stroma in vivo. Matrix metalloproteinases were shown to be expressed both in the epithelium and stroma of the invading terminal end buds, suggesting that these enzymes enable epithelial invasion into the mammary fat pad (Talhouk et al. 1991; Simian et al. 2001; Wiseman et al. 2003; Mori et al. 2009; Mori et al. 2013). To dissect whether matrix metalloproteinase 14 (MMP14) is a key signaling molecule in branching morphogenesis, we utilized 3D culture models comprised of primary mammary organoids and mammary epithelial cell (MEC) line for our study. Motivated by data from a genetic MMP14 mutant mouse, we were able to use our 3D models to uncover reciprocal pathways required for mammary branching morphogenesis (Yana et al. 2007). We found that MMP14 is required for invasion of MECs through stroma and these interactions drive MEC invasion through a collagen-1 microenvironment. Additionally, we identified signals downstream of MMP14 and uncovered the interaction between MMP14 and integrin-β1 (ITGB1) that is essential for MEC invasion to occur. Given the high expression levels of MMP14 in breast cancer, we proposed that the mechanisms we uncovered for branching of normal mammary epithelium are also relevant to the invasion of breast cancer cells through the stroma that surrounds the mammary carcinoma (Mori et al. 2013).

From using 3D models to study development and the interactions of MMP14 in branching morphogenesis, it became apparent that we could further utilize this assay as a system to elucidate the means by which cells become cancerous. Utilizing an elaborate genetic backcross study, we sought to analyze the genetic contributions involved in mammary cancer susceptibility in response to a stimulus such as low dose radiation. Using our 3D culture model and a genome-wide single nucleotide polymorphisms (SNPs) analysis, we revealed how treatment with ionizing radiation led to interactions with the genetic loci and identified TGF-β1 as a factor regulating cancer susceptibility. Our *ex vivo* models allowed us to assess the particular signaling components that provide resistance to cancer risk thus opening possible new avenues to identify individual risk for environmental exposure and cancer (Zhang P*, Lo A* et al. 2015).

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Nomenclature

2D	two dimensional
3D	three dimensional
ADAMTS	a-disintregrin-and-metalloproteinase-with-thrombospondin-like-motifs
AFM	atomic force microscopy
AWRC	animal welfare and research committee
β-GAL	beta-galactosidase
BMP-1	Bone morphogenetic protein 1
BSA	bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
dCAT	deleted catalytic domain
DMEM	dulbecco's modified eagle's medium
DMEM / F12	dulbecco's modified eagle's medium: ham's F12 nutrient mixture
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
EP	early pregnancy
ER	estrogen receptor
ERα	estrogen receptor alpha
F1	first filial generation
FACIT	fibril associated collagens with interrupted triple helices
FBS	fetal bovine serum
FGF2	fibroblast growth factor 2

IHC	immunohistochemistry
ITGβ1	integrin beta 1
ITS/PS	insulin transferrin selenium / penicillin streptomycin
LEP	luminal epithelial cell
LDIR	low-dose ionizing radiation
LN1	laminin-111
LP	late pregnancy
lrECM	laminin-rich extracellular matrix
MACIT	membrane associated collagens with interrupted triple helices
MAPK	mitogen-activated protein kinase
MEP	myoepithelial cell
MMTV	mouse mammary tumor virus
MMP	matrix metalloproteinase
MMP3	matrix metalloproteinase 3
MMP9	matrix metalloproteinase 9
MMP14	matrix metalloproteinase 14
MP	mid pregnancy
MT	membrane-type
MT-MMP	membrane type matrix metalloproteinases
MULTIPLEXIN	multiple triple helix domains with interruptions
PBS	phosphate buffered saline
PFA	paraformaldehyde
RT	room temperature
RT-PCR	reverse transcription polymerase chain reaction
shRNA	small hairpin RNA
siRNA	small interfering RNA

SMA	smooth muscle actin
TEB	terminal end bud
TGF-α	transforming growth factor alpha
TGF-β	transforming growth factor beta
V	virgin
WT	wild type

Acknowledgements

My work and training throughout my graduate career would not have been possible with out the support of a plethora of individuals in my life. I would like to greatly thank the Comparative Biochemistry graduate department and the Department of Energy for supporting my graduate work throughout the years. Secondly, this work would have never come to fruition without the continued support from my mentor Dr. Mina J. Bissell, she has been the driving force from the beginning and my graduate work would have never been possible without her. Next, I would like to thank my peers in the Bissell laboratory for providing me mentorship and assistance. Specifically, I would like to thank current and previous members of the Bissell lab, namely I would like to thank Hidetoshi Mori, Jamie Inman, Joni Mott, Aaron Boudreau, Saori Furata, Jamie Bascom, Rick Schwartz, Kandice Tanner, Rana Mroue, Cyrus Ghajar, Alexandre Bruni-Cardoso, Doug Brownfield, and Negest Williams. Lastly, I would like to thank my parents Kam and Shirley, who supported me throughout the years.

I want to thank everyone who made it possible for me to complete this degree, this includes all my friends who gave me the strength to keep pushing forward and especially my sister Elaine who instilled the determination in my spirit to never give up. Thank you everyone, this would have never been possible without you and I am eternally grateful.

Chapter 1 – Hypothesis and Specific Aims

1.1 Introduction

The mammary gland has intrigued biologists for decades spurring numerous research proposals on its development. The mammary gland is a branched tubulo-alveoar organ with a cyclic existence that changes during reproductive periods (D. G. Blackburn 1993; Neville & Daniel 1987). The mouse mammary system is one of the most extensively studied models systems available. Consisting of a tubular structure, the main function of the mammary gland is to produce and secrete milk. The central unit of the mammary gland, the alveoli is comprised of an inner layer of luminal epithelial cells and an outer layer of myoepitelial cells that are in contact with the basement membrane (Gudjonsson et al. 2002). Functional only in the adult life, mammary gland development begins in the embryo during which the epithelium invades through the mescenchyme in a well-orchestrated fashion (Robinson 2007). Subsequently, in postnatal mice a rudimentary ductal tree has been established during embryogenesis in the mammary fat pad and ductal invasion will continue till the epithelium reaches the end of the fat pad.

These developmental programs executed within the mammary gland are unique processes reflecting the intricacies of the tissue. Owing to the complex milieu of signals necessary for the distinct stages of development and remodeling, analysis of the dynamic nature of the mammary gland is a daunting task (Sternlicht 2006). Several questions remain regarding the maintenance and development of the mammary gland, particularly how the epithelium invades into the fat pad during development and what cues are involved in regulating the growth and elongation of epithelial invasion. A few questions of particular interest for this dissertation are 1) What roles does matrix metalloproteinases play in mammary epithelial architecture? 2) Is there a non-catalytic role for matrix metalloproteinases in mammary epithelial function and architecture? 3) How does TGF-beta contribute to mammary gland development with emphasis to alveologenesis? To answer such questions, techniques and quantitative methods are necessary which will be explored in depth in this dissertation.

1.2 Objective

This dissertation seeks to understand the dynamic role epithelial cells play during development. Using the mammary gland as a model, within this dissertation three main hypotheses exist and will be explored within each subsequent chapter. To begin, we will first seek to dissect out the complex milieu of signals that are necessary for mammary development, focusing in on a particular developmental stage, the maintenance of the acinus. Specific Aim I. will detail a model system to study the role of matrix metalloproteinases during mammary epithelial branching morphogenesis. We hypothesize that only in such a model system can we discern the complexity of matrix metalloproteinases and their ultimate role during the formation of branched structures. Next, we progressed to studying the role of matrix metalloproteinases in mammary epithelial ductal formation. To that end, we hypothesize that there is a non-catalytic function for matrix metalloproteinase 14, allowing mammary epithelial cells to for ductal

branches without catalytic activity. Specific Aim II details an approach to dissect the non-catalytic activity from matrix metalloproteinase 14 and identify the molecular signaling involved in non-catalytic ductal branching. Finally, from the previous study we continued our focus on alveolar formation and investigate the involvement of TGF-beta in mammary alveologenesis and its involvement in cancer susceptibility. We hypothesize that an increase in endogenous TGF-beta can decrease cancer risk by suppressing exogenous signals that are upregulated in cancer. Specific Aim III details an assay using a three-dimensional culture system as a model to assess the significance of increased endogenous TGF-beta expression.

1.3 Specific Aims

1.3.1 Specific Aim I. – Three-dimensional culture models for mouse mammary epithelial cells

In this aim, we thought to provide a methodology for analyzing mouse mammary epithelial cells in a more physiological context. Towards that end, we developed a three-dimensional culture model to simulate and visualize the formation of either mammary ductal branching or mammary alveolargenesis. This model will allow us to recapitulate the structures observed within the mouse in culture, providing us the ability to manipulate the conditions and observe changes within a physiological context.

1.3.2 Specific Aim II. – Role of non-catalytic activity of matrix metalloproteinases in mammary epithelial function and architecture

For this aim, the goal was to determine the impact of the non-catalytic activity of matrix metalloproteinase 14 in mammary branching. Using an in vitro model of mammary gland branching, we used three-dimensional type-I collagen gels to model how functions during epithelial invasion. First, by measuring the stiffness of various collagen gel densities we use either sparse collagen (less rigid) or dense collagen (more rigid) gels to assess matrix metalloproteinase 14 proteolytic activity. Lastly, to look for a molecular mechanism by which the non-catalytic activity of matrix metalloproteinase 14 is acting upon we utilized various tools to manipulate the proteolytic activity.

1.3.3 Specific Aim III. – The involvement of TGF-beta in mammary development and carcinogenesis

The final aim seeks to probe the role of TGF-beta in mammary development and its involvement in cancer resistance. Partially inspired from the experiments from the previous aim, we wished to utilize the branching morphogenesis assay as a metric for invasion and responsiveness to exogenous growth signals. Furthermore, we instead utilized alveolar formation in three-dimensional culture for testing the sensitivity of growth factor response. Finally, we focused our efforts on identifying changes in the signaling cascade and expression of endogenous protein leading towards exogenous growth factor insensitivity.

1.4 Organization

This document outlines three focused yet interlocking studies that, taken together, elucidate the interactions of the mammary gland stroma on development with focused investigation on matrix metalloproteinases and TGF-beta function. As such, these studies separate into distinct individual chapters detailing how the study was conceived, designed, developed and executed towards understanding mammary gland development in relation towards branching and alveolar formation.

Charpter 2

This first chapter will provide a general review of the relevant literature, both classic and current about pertinent information with regards to the mammary gland. As this dissertation details work that is extremely interdisciplinary background information is necessary thus this chapter will be segmented into four parts. A significant portion of the introduction will focus on the mammary gland as an experimental model. This information will be extremely pertinent for the entire dissertation. Following this section, there will be a section detailing the extracellular matrix and its importance. The next two sections will cover matrix metalloproteinases and transforming growth factor beta (TGF-beta) respectively since both are the main topics of study for Chapters 3, 4, and 5.

Chapter 3

This is the first chapter diving into our experimental work. We start with a more focused look into the creation of culture models for investigating mammary gland developmental. Emphasizing models that recapitulate the complex milieu observed *in vivo* we give a detailed description of the application of three-dimensional cultures as a system that replicates the developmental processes seen within the mouse. Using various matrices, we can tailor the microenvironment to simulate various developmental cues. The first half of the chapter will look into developing a model to simulate alveolargenesis, a process observed at the buds of the mammary gland. The subsequent part of this chapter will then focus on branching morphogenesis, a process of invasion into the mammary fat pad, a critical event for mammary gland development. By developing ex vivo models as tools we can fine tune the system to investigate minute processes that we are unable to do *in vivo*.

Chapter 4

Chapter 4 focuses upon the role of matrix metalloproteinase 14 and its non-catalytic function during mammary branching. The initial thought was to understand more in depth the involvement and catalytic function of matrix metalloproteinase 14 in mammary ductal branching, but once discovering a novel non-catalytic function of matrix metalloproteinase 14 our attention switched directions. We found that when mammary epithelial cells are cultured in a sparse type-1 collagen matrix they do not rely on matrix metalloproteinase 14 catalytic function to degrade and break down the type-1 collagen matrix to form branched structures. We were able to conclude that matrix

metalloproteinase 14 catalytic function was dispensable but β1-integrin and matrix metalloproteinase 14 were both essential for branching within type-1 collagen gels.

Chapter 5

Chapter 5 focuses upon the role of the extracellular matrix on cancer susceptibility. Using a distinct wild-derived inbred strain of mice less susceptible to cancer, we sought to examine the functional differences against a highly susceptible mouse strain. From there we began out basic studies on the various differences exhibited between the two mouse strains and identified a distinct difference within the mammary gland. We found that within the wild-derived strain, there exhibited differences within the developmental cues. The first half of this chapter will focus on the basic fundamental differences observed between the two strains of mice. The second half of the chapter will elucidate a functional assay to dissect out the intricacies between the mammary epithelial cells. This half will round out the chapter identifying a particular molecular involved in the distinct phenotypes observed within the two strains of mice and suggest reasoning to why the wild-derived strain is less susceptible to cancer.

Chapter 6

In this chapter we summarized the conclusions from the previous three key chapters and identify crucial points for further investigation. Additionally, potential future steps will be brought up and discussed within this chapter.

Chapter 2 – Background and significance

Within the past century, the scientific community sought to investigate the maintenance of life in all shapes and sizes from ants to zebras and antelopes to zebrafish. A great deal of information has accumulated from the studies of all these life forms, but many questions still remain to be answered. With the knowledge the scientific community has accumulated thus far, many unanswered questions remain about the relationship between the extracellular matrix and cells, as a result the answers to many broad questions remain: What are the underlying interactions between the extracellular matrix and the surround cells? Is there a dynamic relationship between the two? How does the genetic variability of humans play into this dynamic relationship? In an attempt to answer these underlying questions, biologists have found that the mammary gland as a unique and useful model for investigation. The unique mechanism by which milk is produced by the organ has been of interest for scientific investigation for at least 400 years(Neville & Daniel 1987). The work done on the mammary gland during the years provided a platform for investigating complex biological interactions. Since this dissertation will focus on the mammary gland, the extracellular matrix, and proteins within the extracellular matrix, a background section describing these components is necessary outlining the known function and regulation about each subject.

2.1 – The mammary gland

2.1.1 – Development

The mammary gland is a unique tissue because it is only functional in adult life. The mammary gland is characterized by the growth of a branched network of epithelial cells (Sonnenberg et al. 1986). The mammary gland consists of two main components: the stroma, which consists of fat and extracellular matrix and the epithelial cells. The development of the mammary gland begins during embryogenesis and continues through stages during adult development(Robinson 2007). The complex milieu of signals during embryonic development controls the precise temporal regulation and location for mammary gland development, displaying an example of complex cell patterning and fate decisions involved in the process. During embryogenesis, mammary development begins with a milk line forming on the dermis of the embryo that will eventually evolve into the mammary placode, which is a thickening of the surface on the ectoderm. The placode will eventually become a mammary gland bud which will lead to a primary mammary sprout and eventually a ductal tree (Robinson 2007). The initial embryonic developmental process has been well documented and is schematically shown in Figure 2.1.

Postnatal development of the mammary gland consists of a cycle of events that include lobuloalveolar differentiation, lactation and involution. Outlined previously, mammary development begins during embryonic development, upon birth the mammary gland continues to develop and during this period before adolescence the mammary cells expand into the fatpad, the mass of densely packed fat and connective tissue comprising a significant portion of the mammary gland. During expansion into the fad pad the epithelial cells grow forming a ductal tree. This process of growth and expansion is an

orchestrated program wired into mammary gland development and involved local and global regulatory processes.

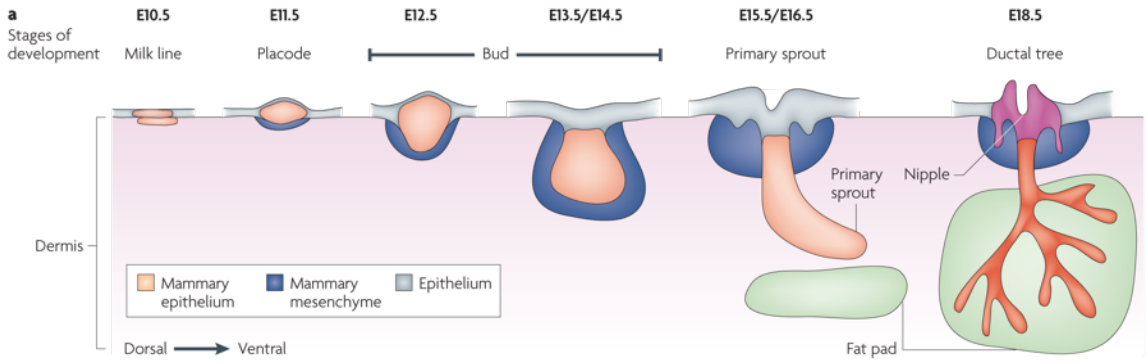

Figure 2.1 Schematic of embryonic mammary gland development. a) Depiction of the various stages of embryonic mammary development beginning with the milk line on E10.5 and ending with the initial ductal tree on E18.5. Over the following 8 days, the mammary epithelium starts to proliferate, organize and remodel the surrounding area before sprouting and invading into the fat pad. (figure used with permission from (Robinson 2007)*).*

During juvenile development, the epithelial portion of the mammary gland increases into the unoccupied area of the fat pad. At the invading front, the mammary epithelial cells form bud like structures. These so-called 'end buds' are comprised of densely packed cells, at which these cells divide at a high rate to advance progression into the fat pad(Hennighausen & Robinson 2005). With certain end buds termed terminal end buds (TEBs), these TEBs have two distinct characteristics: 1) a cap layer of cells on the invading front and 2) a more central located pack of body cells in the bud. These end buds have a club-shaped structure with regards to their visual appearance. During both the embryonic state and the pubertal state, the buds are under the influence of the circulating hormones within the body(Robinson 2004; Hennighausen & Robinson 2005). After several weeks the TEBs will have elongated and invaded to the end of the mammary fat pad thus generating the mature ductal tree of the mammary gland. By examining the end bud structure, you will notice various components to the unit that drives ductal morphogenesis. On the basal surface of the end bud, the outermost cells surrounding the invading front are the cap cells. These cap cells are distinguishable from other mammary gland cell types due to their absence of differentiation and protein markers such as P-cadherin (Slorach & Werb 2003; Srinivasan et al. 2003; Williams & Daniel 1983; Daniel & Strickland 1995). Within the end bud body, there is a distinguishable differentiation of cells composed of a multi-layered epithelium that is comprised of tightly packed luminal cells. Still, it remains unclear whether or not body cells are derived from cap cells, but there is evidence to suggest that cap cells differentiate into myoepithelial cells. These myoepithelial cells are continuous around the cap cell layer and form the outer most layers surrounding the end bud (Williams & Daniel 1983). A composite drawing of an end bud is

well detailed in Figure 2.2. Although one of the major structures of interest during mammary gland development, end buds are not the only component regulating branching and invasion of the mammary epithelium into the fat pad (Williams & Daniel 1983).

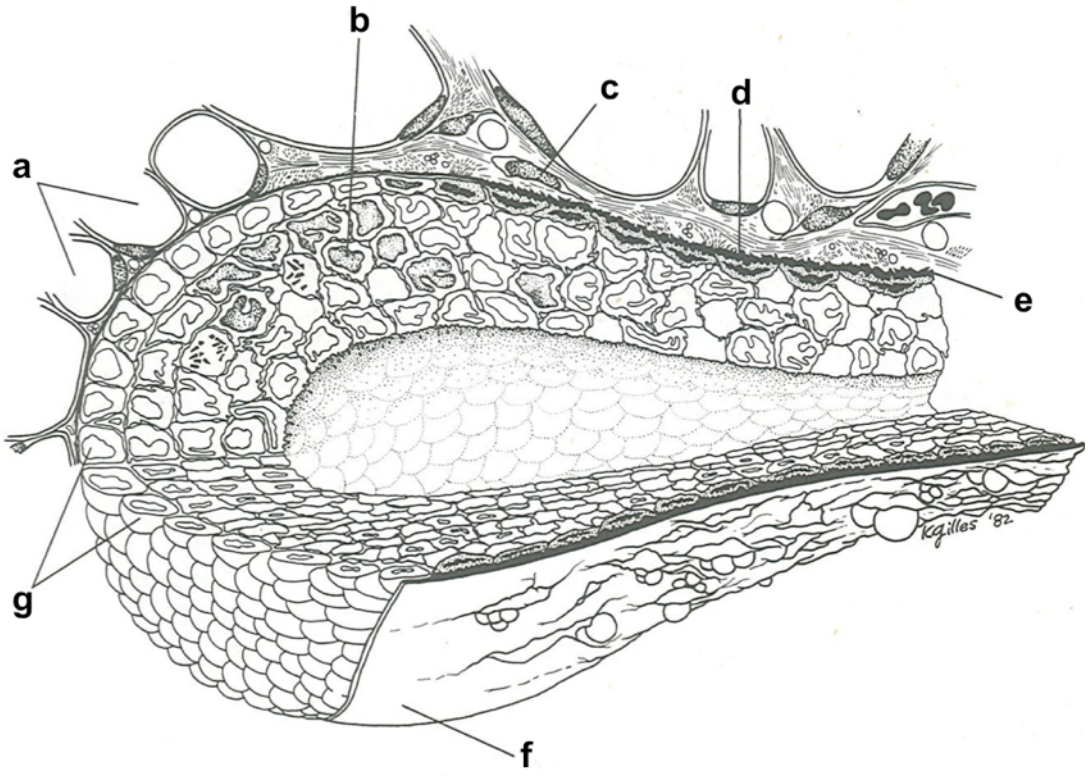

Figure 2.2 Composite drawing from an electron microscope analysis of a mammary end bud. a) adipocytes against the end bud cap cells at the tip. b) mitosis of the body cells. c) fibroblasts along the periphery along with fibrous components. d) myoepithelial cells along the neck region of the end bud. e) basal lamina overlying the myoepithelial cells. f) basal lamina near the invading front. g) cuboidal cap cells at the invading front that will later differentiate into myoepithelial cells. (figure was adapted and used with permission from (Williams & Daniel 1983)*)*

During the juvenile stage, hormones such as estrogen and progesterone regulate the proliferation of mammary cells and the control of ductal outgrowth and alveolar expansion, respectively. In estrogen receptor alpha (ER-α) knock-out mice, mammary gland ductal branching is inhibited (Bocchinfuso et al. 2013). During mammary gland development in early pubertal mice, the mammary gland ducts increase their growth significantly. The end buds beginning to invade into the fat pad and the gland's growth rate now exceeds the isometric rate at which the animal is growing at (Rillema 1994). Mitotic activity remains high until the end buds reach the periphery of the fat pad. Once reaching the periphery of the fat pad, the end buds become less mitotic and ductal elongation ceases. At this stage the mammary gland becomes a mature gland and will bifurcate along the main ducts and form side-branches to fill the fat pad. Once a gland has matured, the animal will have

matured also and become fertile for pregnancy. During pregnancy, the mammary gland undergoes another developmental change. During this stage, the mammary gland exhibits rapid proliferation of the epithelium in preparation for milk production. Along the mammary ducts, alveolar structures from. After birth, the mammary gland begins manufacturing milk, which is generated by the luminal epithelial cells within the alveoli. The milk is accumulated within the alveoli and then secreted thru the ducts and finally to the nipple. When nursing is complete the mammary gland regresses to a state prior to lactation and pregnancy resembling the developmental stage of a mature gland. This process is called involution. Involution involves the suspension milk production and the collapse of the mammary alveoli. Controlled cell death of the lobular alveoli within the mammary gland causes the mammary to return to it's pre-pregnancy state (Neville & Daniel 1987). The development of the mammary gland through these stages is depicted in Figure 2.3.

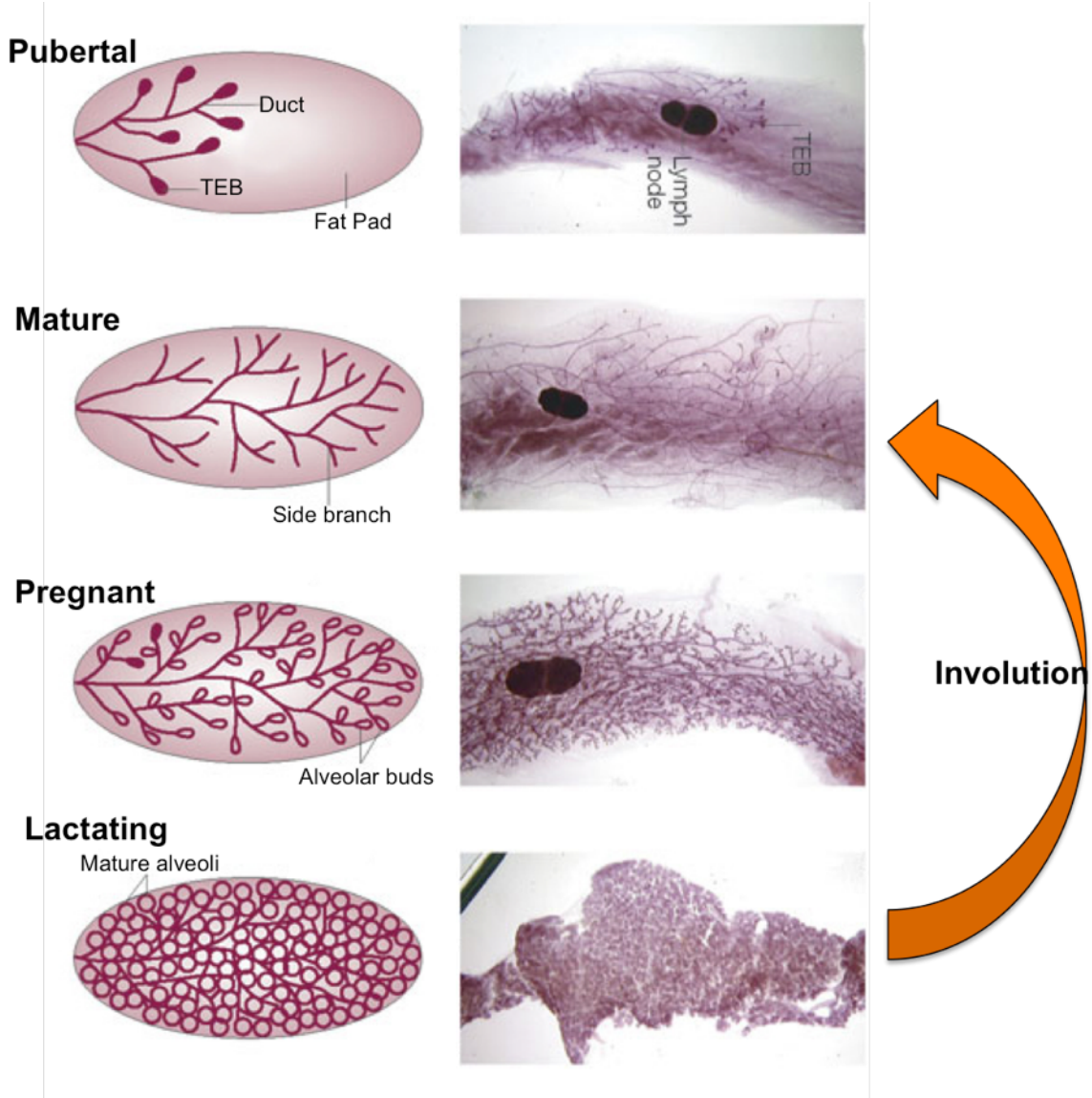

Figure 2.3 Schematic of mammary gland development at various states. After birth, the pubertal mammary gland displays a rudimentary structure with TEBs leading the elongation and invasion. After the gland matures, the TEBs disappear and side-branches occur to fill the entire fat pad. Upon pregnancy, the ends of the branches develop to form alveolar buds, which will eventually produce milk. After birth, the lactation program of the mammary gland occurs, at this stage the alveolar buds generate milk, which will then be secreted through the ducts and ultimately through the nipple to feed the newborn. (figure adapted and used with permission from (Hennighausen & Robinson 2005)

2.1.2 – An experimental model

The mouse mammary model has been used throughout the past century by hundreds of researchers. The ability to use the mouse as a model system stems from the origin of the first inbred mouse strain generated by C.C. Little in the early 1900s. As an artificial creation, the laboratory mouse is a domesticated “fancy mouse” that arose from many years of trading by mouse fanciers and inbreeding. C.C. Little developed the first inbred mouse strain DBA. This led to the more development and generation of many inbred mouse strains throughout the decade. The development of the inbred mouse strain allowed researchers to study with rigor and specificity the pathology of mammary tumors due to the reliability of inbred strains to reproduce results. With advances in genetically engineered mice, researchers were able to alter the parent genome incorporating mutations, transgenes, and deletions to help elucidate basic biological questions within mammary gland biology. With the discovery of MMTV, mammary gland biologists were able to identify the particular gene networks associated with the tumor virus and provide insights into the progressive nature of mammary tumors (Bittner et al. 1945; Callahan & Smith 2000).

The developmental programs described previously executed within the mammary gland are unique processes reflecting the intricacies of the tissue. Owing to the complex milieu of signals necessary for the distinct stages of development and remodeling, analysis of the dynamic nature of the mammary gland is a daunting experimental task(Sternlicht 2006). Thus surrogate methods of investigating the processes involved in mammary gland function and dysfunction are necessary. Employing a surrogate ex vivo assay approach, we can faithfully recapitulate physiological processes and generate multiple experimental replicates from the tissue from a single mouse. Furthermore, by using an ex vivo approach can be more easily probe the signaling mechanisms that occur during the different morphogeneic processes.

It has been known for some time that once cells are taken from a native tissue environment and placed in traditional two-dimensional (2D) culture they loose functional performance and relevant morphology (D. M. Bissell 1981; M. J. Bissell 1981). We have shown that in 2D culture, mammary epithelial cells are unable to induce tissue-specific gene expression. It is only in the presence of signals from the extracellular matrix will tissue specific gene expression occur and allow for the formation of structures that are similar to those observed in vivo (Barcellos-Hoff et al. 1989; Streuli et al. 1991; Streuli et al. 1995; Myers et al. 1998; Novaro et al. 2003; Xu et al. 2009; Spencer et al. 2011). Over the past several decades, we have designed ex vivo three-dimensional (3D) culture assays that recapitulate the morphogenic programs of alveologenesis as well as ductal invasion and elongation that allow for the study of the mammary gland (Hirai et al. 1998; Simian et al. 2001; Fata et al. 2007). Using these 3D culture models, others, and we have been able to systematically define biochemical and environmental signaling cues that are important in mammary gland biology.

2.2 – The extracellular matrix

The extracellular matrix can be defined in many ways by different investigators. It can be defined as an acellular material that connects cells within a given tissue or it can be called a substance that provides mechanical support and physical strength to tissues and organs (Neville & Daniel 1987; Ayad et al. 1998). The extracellular matrix is quite a diverse mixture of proteins providing solely physical support for simplistic organisms or connective substrata for more complex ones. The extracellular matrix should not be viewed only as a material that yields support but it is quite clear that the matrix provides influence in both the behavior of cells via their gene expression and their interaction with other cell types (Streuli et al. 1991; Streuli et al. 1993). The common components of the extracellular matrix, Collagen, is a principal fiber and is part of a family of highly specialized glycoproteins which there are now 28 types that have been identified encoded by 43 genes. Collagen has a complex structure, in addition to some being encoded by multiple genes such as type-I collagen, there are close to 30 types of collagen. Type-I collagen is the most abundant in our bodies with over 90% of the collagen within our bodies consisting of this type. Not limited to only collagen, other extracellular matrix proteins such as proteoglycans, which are a diverse family of proteins characterized by having one or more glycosaminoglycans attached on its side-chain are important components in the extracellular matrix. Lastly, another important component in the extracellular matrix are glycoproteins, these molecules function as major structural elements within the matrices but have other physical aspects to them in regards to modulating cell signaling.

2.2.1 – Collagen

Collagen is a major component of the extracellular matrix and constitutes a highly differentiated family of glycoproteins. For a protein to be classified as a collagen molecule it has to be an integral part of the extracellular matrix. Currently 28 types of collagen molecules have been identified, of which at least four comprise the majority of collagen types within a human being (Matthew D Shoulders 2009). Collagen molecules have multiple components that make up the triple helix structure. The triple helix structure is constructed from three polypeptide chains, each with a helical configuration. The helical structures vary between collagen types but they can either be continuous or interrupted with non-helical domains. Collagen molecules are classified in five various types: fibrillar, network non-fibrillar, fibrils associated collagen with interrupted triple helix (FACIT), membrane associated collagen with interrupted triple helix (MACIT), and multiple triple-helix domains and interruptions (MULTIPLEXINs). Listed in Table 2.1 are the collagen types along with their classification and composition (Matthew D Shoulders 2009).

Fibrillar forming collagens are usually referred as the ‘classical’ collagen because fibrillar collagens account for 80-90% of all the collagen in the body. The synthesis of fibrillar collagen begins with the synthesis of the precursor procollagen proteins that comprise the collagen molecule. The procollagen proteins contain a non-collagenous C-terminal propeptide and an N-terminal propeptide. The presence of the propeptide on the procollagens prevents premature assembly of the collagen molecules into fibrils. These

procollagen precursors then are secreted and cleaved extracellularly by proteinases during fibrillogenesis giving rise to a mature collagen helix consisting of the triple helix structure. The triple helix structure is depicted in a cartoon from a high-resolution crystal structure in Figure 2.4.

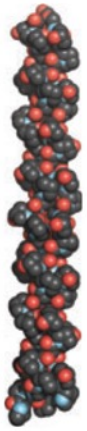

Figure 2.4. High-resolution crystal structure of a collagen triple-helix formed from (ProHypGly)$_4$ [Protein Data Bank (PDB) entry 1cag](Bella et al. 1994). *Figure was used with permission and adapted from* (Matthew D Shoulders 2009).

Another important type of collagen is the network-type of collagen, referred usually as Collagen IV which is considered a basement membrane collagen due to its integrated network of several matrix molecules that form the extracellular matrix (ECM) that constitutes the interface between tissues (Khoshnoodi et al. 2008). Collagen IV is found exclusively in the basement membrane and is involved in processes such as differentiation, cell adhesion, and migration. Collagen IV is made up of six highly homologous chains with each chain containing three structurally distinct domains; an amino-terminal domain rich in cysteine and lysine amino acids, a collagenous triple repeat of 1,400 residues comprised of Gly-Xaa-Yaa, and lastly a 230 long amino acid carboxy terminal non-collagenous domain, termed NC1 domain (Khoshnoodi et al. 2008). Crystallography analysis of the NC1 hexamer of collagen IV has provided a detailed structural information analysis on the interaction between NC1 domains, depicted in Figure 2.5.

Looking at the assembly of collagen, focusing our view on collagen-1, the initial assembly of the collagen-1 triple-helical structure is regulated by the propeptide segments of the collagen molecule through the processing and cleavage of the propeptide segments by enzymes. Looking at the assembly of fibril-forming collagens, the C-terminal propeptides are processed through the enzymatic cleavage by bone morphogenetic protein 1 (BMP-1)/tolloid proteinases (Mienaltowski & Birk 2014). Processing of the N-terminal pro-peptide is done through an a-disintegrin-and-metalloproteinase-with-thrombospondin-like-motifs family (ADAMTS) as well as BMPs. Specific enzymes have preferential

selection for certain fibril collagens, and processing of the collagen can be both complete and incomplete, with the latter leaving an unprocessed terminal end thus retaining the pro-peptide form. This incomplete processing has been implicated in the regulation of fibrillogenesis (Rousseau et al. 1996). Once processing of the immature collagen is complete, the collagen molecules will self-assemble to form striated fibrils with a periodicity of 67nm (Mienaltowski & Birk 2014).

With collagen-1 being the most abundant type of collagen in the human body, here on we will focus on fibril collagens and their structure and function. After processing of the pro-peptide form and assembly into striated fibrils, each fibrillar collagen molecule will have an approximate length of 300nm with a diameter of 1.5nm. Within the collagen fibril, the molecules are staggered in a N-terminal to C-terminal pattern giving rise to a D-periodic repeat (Mienaltowski & Birk 2014).

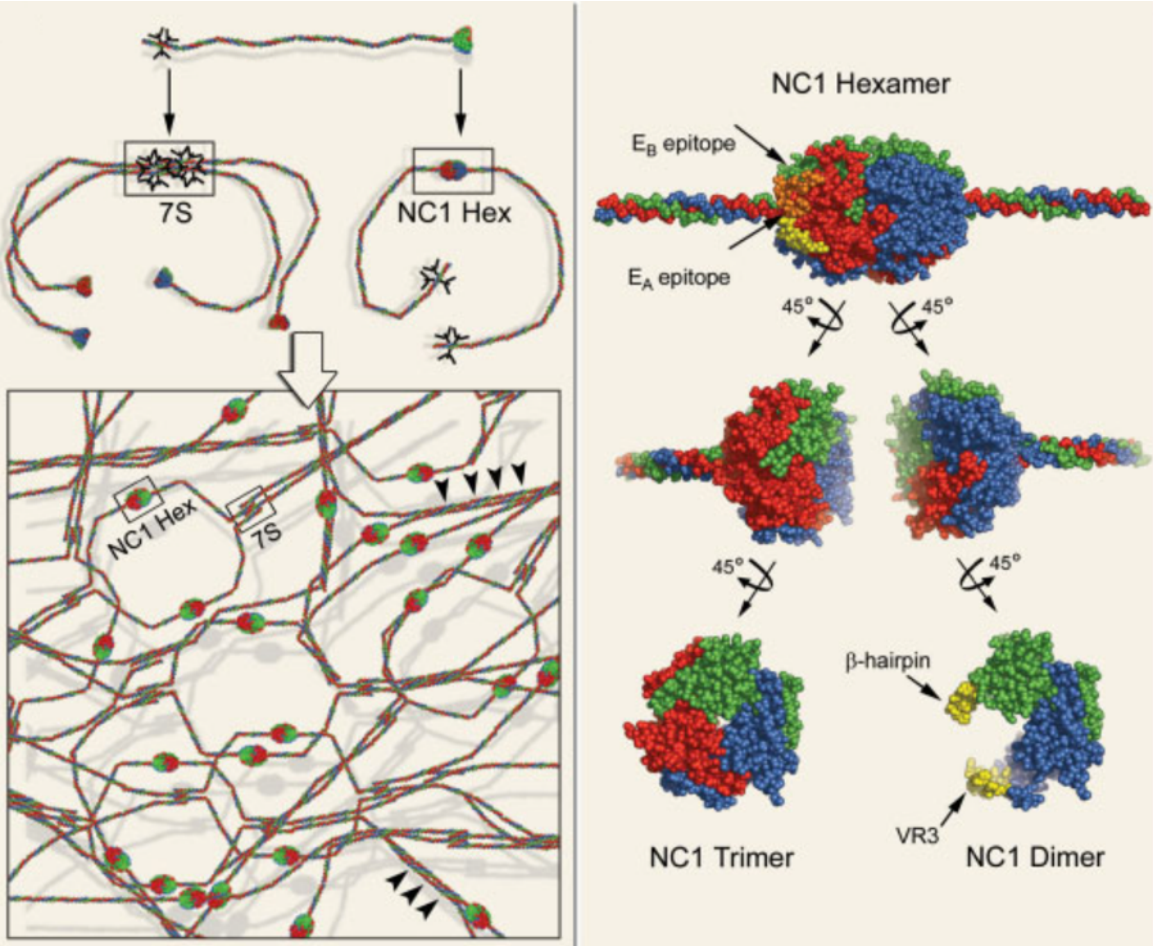

Figure 2.5. A generic representation of the structural NC1 domain from a collagen IV molecule. The crystal structure illustrates the end-to-end interaction between two NC1 trimers to form the NC1 hexamer. Figure was used with permission and adapted from (Khoshnoodi et al. 2008)

Type	Class	Composition	Distribution
I	Fibrillar	α1[I]2 α2[I]	Abundant and widespread: dermis, bone, tendon, ligament, mammary gland, etc.
II	Fibrillar	α1[II]3	Cartilage, vitreous
III	Fibrillar	α1[III]3	Skin, blood vessels, intestine
IV	Network	α1[IV]2 α2[IV] α3[IV]α4[IV]α5[IV] α5[IV]2 α6[IV]	Basement membranes
V	Fibrillar	α1[V]3 α1[V]2 α2[V] α1[V]α2[V]α3[V]	Widespread: bone, dermis, cornea, placenta
VI	Network	α1[VI]α2[VI] α3[VI] α1[VI]α2[VI] α4[VI]	Widespread: bone, cartilage, cornea, dermis
VII	Anchoring fibrils	α1[VII]2 α2[VII]	Dermis, bladder
VIII	Network	α1[VIII]3α2[VIII]3 α1[VIII]2 α2[VIII]	Widespread: dermis, brain, heart, kidney
iX	FACIT	α1[IX]α2[IX]α3[IX]	Cartilage, cornea, vitreous
X	Network	α1[X]3	Cartilage
XI	Fibrillar	α1[XI]α2[XI] α3[XI]	Cartilage, intervertebral disc
XII	FACIT	α1[XII]3	Dermis, tendon
XIII	MACIT	-	Endothelial cells, dermis, eye, heart
XIV	FACIT	α1[XIV]3	Widespread: bone, dermis, cartilage
XV	MULTIPLEXIN	-	Capillaries, testis, kidney, heart
XVI	FACIT	-	Dermis, kidney
XVII	MACIT	α1[XVII]3	Hemidesmosomes in epithelia
XVIII	MULTIPLEXIN	-	Basement membrane, liver
XIX	FACIT	-	Basement membrane
XX	FACIT	-	Cornea (chick)
XXI	FACIT	-	Stomach, kidney
XXII	FACIT	-	Tissue junctions
XXIII	MACIT	-	Heart, retina
XXIV	Fibrillar	-	Bone, cornea
XXV	MACIT	-	Brain, heart, testis
XXVI	FACIT	-	Testis, ovary

XXVII	Fibrillar	-	Cartilage
XXVIII	-	-	Dermis, sciatic nerve

Table 2.1. Various collagen families, detailing the type, the associated contribution and the distribution within the body, table was generated using information from (Ayad et al. 1998; Matthew D Shoulders 2009).

Collagens have many different structures and functions that vary depending on the collagen type. Focusing on fibril collagen, more specifically collagen-1, which is the most abundant collagen in the human body, its main function is to provide structural rigidity and form to organs and tissues. Mutations in the COL1A1 gene leads to serious implications such as osteogenesis imperfecta or osteoporosis both are conditions that negatively affect bone strength (van Dijk et al. 2011). Focusing our analysis on the mammary gland, indeed collagen-1 is responsible for structural rigidity and support of the tissue but it also provides separation between the highly organized epithelium and the stroma (Schedin & Keely 2011). This mechanical stiffness provided by the collagen-1 matrix provides an excellent scaffold to direct organization of the epithelium leading to alignment of the mammary ducts (Brownfield et al. 2013). While it is well understood how collagen-1 primarily functions structurally, the secondary functions have more recently been elucidated and it has become clear that collagen-1 serves not only as a molecule to provide structure and rigidity to tissues but to provide direction and development of the tissue also. As our understanding of collagens increases thus will our knowledge of the ECM as a whole.

2.2.2 – Laminin

Laminin is a type of adhesive glycoprotein that is within the extracellular matrix (Ayad et al. 1998). In mammals there are at least 15 types of laminins while in invertebrates they have been found to possess one or two types (Miner & Yurchenco 2004). The increase in the number of laminin types poses interesting questions about the functional diversity of laminins between mammals and invertebrates. Each laminin is a heterotrimer, where each individual polypeptide chain is joined into a long coiled-coil to produce a molecule resembling a cruciform like structure with one long arm and three short arms. Laminins are formed through the combinations of several α, β, and γ subunits. Each different laminin genes coding for each subunit increases the complexity for the various laminin types due to various RNA splicing. The structure of laminin is made up of three distinct chains encoded by specific genes. Forming a cruciform structure, the laminin with three short arms and a long arm; the long arm is formed from a triple-coiled coiled structured from all the subunits. Each individual short arm is comprised of the N-terminal regions of each individual subunit. All laminin chains share a similar domain structure. Domains I & II are made of up of a series of heptad repeats with predicted α-helical confirmation which are formed from the long arm. Domains I & II are located at the carboxyl-end of the β and γ chains and in a similar region as the α chain. Domains III & V consist of around 50 amino acids of homologous repeats of that are rich with glycine residues along with eight cysteine residues arranged in a cysteine residue motif resembling that of epidermal growth factor (EGF) and transforming growth factor alpha (TGFα). Domains IV & VI are

the globular regions of the short arms with laminin α1, α2 and α5 containing an additional domain, IIIa and IVa. There are additionally some laminins that contain truncated forms such as α3a, α4, β3, and γ2 which are missing certain domains (Miner & Yurchenco 2004; Ayad et al. 1998). Listed on Table 2.2 are the various know laminin types and their associated trimer composition.

Laminin has many functions; a main function is to provide a structural scaffold for cells. Other identified functions of laminins include maintenance of cell polarity, generation of barriers of between tissue compartments, organization of cells into specific tissues, tissue specific function, apoptosis, and anoikis (Miner & Yurchenco 2004; Streuli et al. 1991; Streuli et al. 1993). A very important activity of laminin is its ability to bind other objects. Numerous studies have been conducted, some of them mapping the specific binding domains but the results of these experiments can be summarized simple with laminin having two distinct binding activities, binding involving matrix assembly or binding involving cell-surface interactions (Miner & Yurchenco 2004).

Laminin Trimers
Laminin-1: α1β1γ 1
Laminin-2: α2β1γ 1
Laminin-3: α1β2γ 1
Laminin-4: α2β2γ 1
Laminin-5: α3Aβ3γ 2
Laminin-5B: α3Bβ3γ 2
Laminin-6: α3β1γ 1
Laminin-7: α3β2γ 1
Laminin-8: α4β1γ 1
Laminin-9: α4β2γ 1
Laminin-10: α5β1γ 1
Laminin-11: α5β2γ 1
Laminin-12: α2β1γ 3
Laminin-14: α4β2γ 3
Laminin-15: α5β2γ 3

Table 2.2. Various laminin types and their associated trimers (adapted from (Miner & Yurchenco 2004)*)*

2.2.3 – Other ECM proteins

Not limited to just collagens and laminins, the ECM is comprised of numerous other molecules that include but are not limited to elastins, proteoglycans, noncollagenous glycoproteins, and microfibrillar proteins. These molecules when combined together form a complex three-dimensional network that provides interaction between the cells in an organ specific manner. Our current understanding of the ECM is that it is a dynamic structure that is in constant flux and generates feedback to the cell and vise versa

producing a fluid environment. Proteins such as hyaluronan, a proteoglycan, is found in the ECM and is essential for cell migration and tissue repair (Chen & Abatangelo 1999). Proteins such as hyaluronan contribute to the overall ECM and provide the important functions and are an integral part of the matrix.

2.3 – Matrix metalloproteinases

Matrix metalloproteinases are a zinc-dependent endopeptidases that were first identified and described over 50 years ago (Gross & Lapiere 1962). These matrix metalloproteinases play a pivotal role in various physiological processes, including but not limited to organ development, tissue remodeling, cell invasion, extracellular matrix remodeling, and inflammatory response (Page-McCaw et al. 2007). Currently, there are 23 matrix metalloproteinases expressed in humans, which are categorized by their enzymatic function, depicted in Table 2.3. The general structure of matrix metalloproteinases remain the same and are characterized by three domains, the pro-peptide domain, the catalytic domain, and the hemopexin-like C-terminal domain linked to the catalytic domain as a hinge. Matrix metalloproteinases are secreted in an inactive confirmation that requires an activator termed convertases to cleave the pro-peptide domain causing the release of the pro domain and opening the enzymatic site (Kessenbrock et al. 2010).

Enzyme	MMP	Human Chromosome	3D Structure (PDB Code)
Collagenases			
Interstitial collagenase; collagenase 1	MMP-1	11q22-q23	Mature protein; 1FBL cat domain; 1CGF, 2TCL, 1AYK, 2AYK, 1HFC, 1CGL, 1CGE, 966C, 3AYK, 4AYK
Neutrophil collagenase; collagenase 2	MMP-8	11q21-q22	Cat domain; 1MNC, 1I76, 1JAO, 1MMB, 1JAN, 1JAP, 1JAQ, 1I73, 1KBC, 1A85, 1A86, 1BZS, 1JJ9, 1JH1
Collagenase 3	MMP-13	11q22.3	Cat domain; 1CXV, 1FM1, 1FLS, 456c, 830c, 1EUB Hpx domain; 1PEX
Collagenase 4 (*Xenopus*)	MMP-18	NA	
Gelatinases			
Gelatinase A	MMP-2	16q13	proMMP-2; 1CK7; proMMP-2–TIMP-2 complex; 1GXD; cat domain; 1QIB, 1HOV, 1EAK; Hpx domain; 1GEN, 1RTG; Fn; 1CXW, 1KS0
Gelatinase B	MMP-9	20q11.2-q13.1	Pro–cat domain; 1L6J; cat domain; 1GKC, 1GKD; Hpx domain; 1ITV
Stromelysins			
Stromelysin 1	MMP-3	11q23	Pro–cat domain; 1SLM; cat domain; D8M, 1ClZ, 1CAQ, 1B8Y, 2SRT, 1HFS, 1SLN, 2USN, 1USN, 1D5J, 1BQO, 1D7X, 1D8F, 1BIW, 1UMS, 3USN, 1UMT, 1BM6, 1B3D, 1CQR, 1G4K, 1G49, 1HY7, 1G05; complex with N-TIMP-1; 1UEA
Stromelysin 2	MMP-10	11q22.3-q23	
Stromelysin 3	MMP-11	22q11.2	1HV5
Matrilysins			
Matrilysin 1; Pump-1	MMP-7	11q21-q22	Cat domain; 1MMP, 1MMQ, 1MMR
Matrilysin 2	MMP-26	11p15	
Membrane-type MMPs			
Transmembrane			
MT1-MMP	MMP-14	14q11-q12	Cat domain in complex with TIMP-2; 1BQQ, 1BUV
MT2-MMP	MMP-15	15q13-q21	
MT3-MMP	MMP-16	8q21	
MT5-MMP	MMP-24	20q11.2	
GPI anchor			
MT4-MMP	MMP-17	12q24.3	
MT6-MMP	MMP-25	16p13.3	
Others			
Macrophage elastase	MMP-12	11q22.2-q22.3	Cat domain 1JK3, 1JIZ
No trivial name	MMP-19	12q14	
Enamelysin	MMP-20	11q22.3	
XMMP (*Xenopus*)	MMP-21	ND	
CA-MMP	MMP-23	1p36.3	
CMMP (*Gallus*)	MMP-27	11q24	
Epilysin	MMP-28	17q21.1	

Table 2.3. List of Matrix Metalloproteinases (adapted from (Visse & Nagase 2003)*).*

Matrix metalloproteinases can be segmented into six categories: Collagenases, Gelatinases, Stromelysins, Matrilysins, Membrane-Type MMPs, and other MMPs. Within collagenases are MMP-1, MMP-8, MMP-13, and MMP-18. Collagenases have the distinct function of primarily cleaving collagens but have the ability to cleave other ECM and non-ECM molecules. MMP-2 and MMP-9 are categorized within the gelatinases group and are also known as gelatinase A and gelatinase B respectively. MMP-2 and MMP-9 have a unique fibronectin domain within their catalytic domain that allows them to bind gelatin, laminin and collagens. Within the stromelysin group, MMP-3 and MMP-10 also referred to ask stromelysin 1 and stromelysin 2 respectively, have very unique substrate specificities. Matrilysins include MMP-7 and MMP-26, also known as matrilysin 1 and matrilysin 2 respectively, are characterized by their lack of a hemopexin domain. MMP-14, MMP-15, MMP-16, MMP-24, MMP-17, and MMP-25 are bundled within the membrane-type MMPs. The four membrane-type MMPs (MT-MMPs) are MMP-14, MMP-

15, MMP-16, and MMP-24, these four can also be referred as MT1-MMP, MT2-MMP, MT3-MMP and MT3-MMP respectively while MMP-17 and MMP-25 are two glycosylphorphatidylinostiol (GPI) anchored MMPs. The last category of MMPs include seven MMPs that are not classified in the previous lists, MMP-12, MMP-19, MMP-20, MMP-21, MMP-22, MMP-23, and MMP-28 have their own unique characteristics (Visse & Nagase 2003).

When thinking about the extracellular matrix and matrix metalloproteinases, there is a controlled balance between the two types of proteins. There exists a dynamic equilibrium between synthesizing new matrix proteins and degradation of them. Taken together the understanding of how MMPs and ECM molecules interact will elucidate how the mammary gland develops and the role of each individual factor.

2.4 – Transforming growth factor beta (TGFβ)

Transforming growth factor beta (TGFβ) is a family of secreted proteins that perform numerous cellular functions that included but are not limited to controlling cell proliferation, cell growth, cell migration, cell differentiation, cell death, and apoptosis. TGFβ is secreted by a number of cell types including epithelial cells, fibroblasts, and macrophages. When these cells secrete TGFβ, it is secreted in a latent form and resides in the ECM of the surrounding cells until it becomes activated. Activation of latent TGFβ can by achieved through the secretion of plasmin or other extracellular protease (Annes et al. 2003). TGFβ exists in at least three isoforms, TGFβ1, TGFβ2, and TGFβ3 with each signaling to various cell receptors. With its multitude of cellular responses TGFβ has been characterized as being a double-edged sword as it can be considered an antiproliferative agent or a proliferative agent during cancer progression (Massagué 2012).

TGFβ are ligands and have specific targets to receptors on the cell surface. These receptors are superficially similar to receptor tyrosine kinases (RTKs) in such a way that both have an extracellular ligand binding domain, a transmembrane domain, and a cytoplasmic domain. The TGFβ receptor functions as a heterodimer leading to the cross-phosphorylation of the reception causing a downstream signaling response that inevitability leads to expression of particular TGFβ activated genes. Termed a 'double-edged sword', TGFβ has two major effects, it can either antagonize or favor tumor progression. During cancer progression, tumor cells resolve the antagonizing affect by learning to evade certain signaling nodes while leaving other intact for a promotion response (Weinberg 2013).

In the context of the mammary gland, TGFβ also plays multiple roles. During mammary gland branching TGFβ can act as a negative regulator for cell differentiation and migration (Nelson et al. 2006). This anti-migration signaling plays an important during development and inhibits the necessary cue for ductal branching (Silberstein et al. 1990). Yet, TGFβ can also increase proliferation in the context of mammary carcinogenesis (Muraoka et al. 2003). The disparate responses to the mammary gland in response to TGFβ is an interesting biological response in determining how this differential response is mediated

during early stage to end stage mammary gland development and cancer progression is an important topic for further investigation.

Chapter 3 – Three-dimensional culture of mouse mammary epithelial cells

3.1 – Introduction

Tissue organogenesis is directed by both intercellular interactions and communication with the surrounding microenvironment. When mammary epithelial cells are isolated and placed in a two dimensional (2D) monolayer culture, the context of the in vivo growth conditions are changed and thus creating an environment less similar to its original. When culture in a three dimensional matrix, in our system we are using a laminin-rich extracellular matrix (lrECM), the fundamental differences between cells cultured on a 2D monolayer and cells in 3D become apparent. A coordination between proliferation and morphogenesis is observed between the two conditions and if properly used can help elucidate the intricacies of mammary cell organization seen in vivo. Using this approach, our laboratory has shown that coordination between proliferation and morphology, measured by basal polarity, and a gain of mammary cell function is displayed when cells are cultured in a 3D matrix (Barcellos-Hoff et al. 1989; Weaver et al. 1997; Liu et al. 2004).

Understanding the pathways regulating the gain of mammary cell function in 3D culture would help the understanding of the processes involved with normal mammary function. Because of the complexity of signals necessary for the distinct functions within the mammary gland, this understanding of the tissue drove the need for better model systems to study mammary cells outside the context of a live animal but with behaviors similar to that within the live animal. In that regard, we have developed in vitro 3D cell culture models to support our understanding of mammary gland function.

During development, the mammary gland undergoes a well-orchestrated process of ductal invasion and elongation culminating in an elaborate ductal tree coinciding with the onset of puberty (Fata et al. 2007; Robinson 2007). During pregnancy, striking morphological and functional changes influenced by the intricate milieu of hormones allows the alveoli, which are the functional units of the mammary gland, to produce milk (Rillema 1994; Medina 1996). The proper function of the alveoli is necessary for lactation to occur. At weaning the gland involutes and the final morphology and function becomes similar to a pre-pregnancy. These developmental programs are processes unique to the intricacies of the mammary gland. Because of the complexity of signals necessary for both the distinct stages of development and remodeling, analysis of the dynamic nature of the mammary gland is a daunting experimental task (Sternlicht 2006). Since both architecture and function are changed during the different developmental stages, by examining mice at particular stages of development, especially during pregnancy can be both time consuming and expensive. Thus surrogate models and methods of investigating the processes involved in mammary gland function are needed. By employing an *ex vivo* assay approach, we can faithfully recapitulate the physiological processes that occur *in vivo* and generate multiple experimental replicates without the need to sacrifice and use multiple animals, thus increasing our ability to do maintain consistency. Furthermore, investigation of signaling mechanisms that occur during different morphogenic processes are more easily studied in *ex vivo* model as opposed to *in vivo* models due to the inherent variability normally observed during *in vivo* studies within mice.

It has been known for some time that once cells are taken from a native tissue environment and placed in a traditional two-dimensional cell culture system, they lose their functional performance and relevant morphology (D. M. Bissell 1981; M. J. Bissell 1981). We have shown previously in many studies that when mammary epithelial cells are culture in a traditional two-dimensional culture, the cells lose their inherent ability to function normally as seen *in vivo* and are unable to induce expression of tissue-specific genes. It is only in the presence of signals from the extracellular matrix that tissue specific gene expression occurs, which allows for the formation of structures that resemble those observed *in vivo*. Over the past several decades, we have designed various *ex vivo* three-dimensional culture assays that recapitulate ductal invasion and elongation, the morphogeneic programs of alveologenesis, as well as functional differentiation of mammary epithelial cells (Barcellos-Hoff et al. 1989; Streuli et al. 1991; Streuli et al. 1995; Myers et al. 1998; Hirai et al. 1998; Simian et al. 2001; Novaro et al. 2003; Nelson et al. 2006; Fata et al. 2007; Xu et al. 2009; Spencer et al. 2011). Using these three-dimensional culture models has allowed us and others to systematically define biochemical and environmental signaling cues that are important in mammary gland homeostasis and gene expression.

3.2 – Methods

3.2.1 – Materials and Instrumentation

Equipment needed for Primary Organoid Isolation and Culture

- Class II Biosafety Cabinet
- Humidified Incubator, 37°C, 5% CO_2
- Orbital Incubator Shaker, 37°C
- Heat block, 37°C
- Water bath, 37°C
- Scanning confocal microscope
- Centrifuge
- Inverted Microscope with Phase Contrast Optics
- Vacuum Flask
- Pipets (1µl to 1ml)
- Pipet-aid
- Razor Blades

Materials needed for Primary Organoid Isolation and Culture

- 48-well Tissue Culture Plate or 8-well Borosilicate Chamber Slide
- DMEM/F12 medium (Invitrogen Cat#11330-032)
- Insulin-Transferrin-Sodium Selenite (Sigma Cat#I1884)
- 1x Penicillin/Streptomycin (Invitrogen Cat#15140-122)
- Transforming Growth Factor alpha (Sigma Cat#T7924)
- Collagenase Type-IV (Invitrogen Cat#17104-019)
- Trypsin (1:250) Powder (Invitrogen Cat#27250-018)

- Deoxyribonuclease I (DNase I) from bovine pancreas (Sigma Cat#D4263)
- Fetal Bovine Serum (Invitrogen Cat#16000-044)
- DMEM/F12 powder (Invitrogen Cat#12400-024)
- Insulin (Sigma Cat#I1882)
- Sterile Phosphate-buffered saline (PBS)
- BSA (Sigma Cat#A2153)
- NaOH (Sigma Cat#221465)
- 60ml Luer-Lok syringes
- 0.45µm Surfacant-Free cellulose acetate syringe filters
- 15ml and 50ml polystyrene conical centrifuge tubes
- 100mm tissue culture dishes
- Growth Factor Reduced Matrigel™ (BD Biosciences Cat# 354231)
- Native Type-I Collagen (Cosmo Bio Co. Cat#KOU-IAC-50)

Solutions needed for Primary Organoid Isolation and Culture

- Digestion buffer (2g/l trypsin, 2g/l collagenase type-iv, 5% (v/v) FBS, 5µg/ml insulin in DMEM/F12 medium)
- ITS/PS medium (DMEM/F12 medium supplemented with 1x Insulin-Transferrin-Sodium Selenite Solution and 1x Penicillin/Streptomycin solution)
- Sterile BSA-PBS solution (5g/l BSA dissolved in PBS)
- Sterile 5mg/ml Insulin solution
- Sterile 0.1N NaOH
- DNase I solution (2U/ul Deoxyribonuclase I dissolved in sterile DMEM/F12 medium)
- Sterile 10X DMEM/F12 solution (resuspend 1L packet in 100ml sterile ddH2O)

Materials and solutions needed for staining and imaging cultures

- Phosphate-buffered saline (PBS) (Sigma Cat#P4417)
- Goat Serum (Invitrogen Cat#16210-072)
- Glycine (Invitrogen Cat#15527-013)
- Triton-X100 (Sigma Cat#X100)
- 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen Cat#D1306)
- 16% (w/v) Paraformaldehyde Aqueous Solution (Electron Microscopy Sciences Cat#15700)
- Glass slides (VWR Cat#48311-600)
- VWR Tissue (VWR Cat#82003-822)
- 4% (w/v) Paraformaldehyde in PBS
- 2% (w/v) Glycine in PBS
- 1:1 Methanol and acetone mixture pre-chilled at -20°C
- 18% (w/v) and 30% (w/v) sucrose solution dissolved in PBS
- Permeablization buffer (PBS containing 0.25% (v/v) Triton-X100)
- Blocking buffer (PBS containing 5% (v/v) Goat Serum and 0.1% (v/v) Triton-X100)
- DAPI solution (PBS containing 0.5µg/ml DAPI)

3.2.2 – Methodology for Isolation of Primary Mouse Organoids

Inguinal mammary glands (the fourth pair of glands) were obtained from four BALB/c mice at eight weeks of age for organoid isolation under an approved protocol from the Animal Welfare and Research Committee (AWRC) at the Lawrence Berkeley National Laboratory. A cartoon illustration of extraction process is depicted in Figure 3.1.

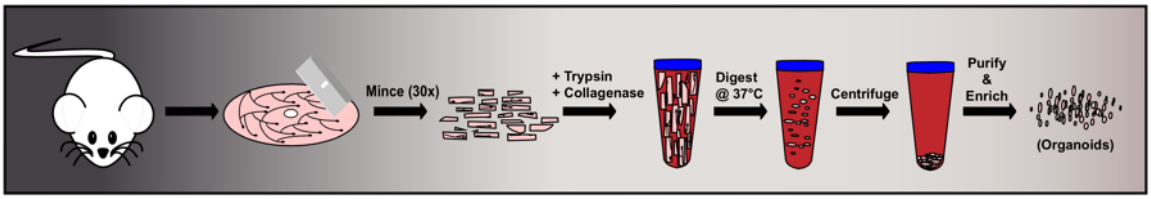

Figure 3.1 Diagrammatic overview of primary mouse organoid isolation in a simplified stepwise manner.

1. Pin the animal down with the ventral surface facing up. With 70% ethanol, wet the animal down to disinfect and prevent hair from dragging.
2. Using forceps, grab the skin anteriorly near the urethral opening and with a pair of scissors cut along the ventral midline from the groin to the chin.
3. Make 15mm incisions from the center of the torso to each side of the animal and using your forceps peel the subcutaneous skin away from the peritoneal cavity exposing the inguinal mammary glands on each side.
4. Using a pair of scissors, remove the lymph nodes from each mammary gland and discard them to prevent the isolation of immune cells.
5. Dissect the mammary glands using forceps and scissors and store the glands in sterile PBS.
6. In a biosafety cabinet remove the mammary glands from the PBS solution and place them in a 100mm tissue culture dish.
7. Using two sterile razor blades pressed together, mince the tissue 30 times to break apart the mammary gland to easily digestible pieces.
8. Using sterile forceps place the minced mammary glands pieces in 50ml of digestion buffer (described above) and incubate in an orbital shaker at 37°C rotating at 100rpm for 30 minutes.
9. After digestion, vortex the tube for 10 seconds to break apart any residual pieces and centrifuge at 1500rpm for 15 minutes at room temperature (RT).
10. There will be three distinct layers visible after centrifugation. The fat layer (top layer), the digestion buffer (middle layer), and the organoids (bottom layer). In addition to the organoids being in the pelleted fraction, there are also organoids within the top fat layer.
11. To recover organoids in the top fat layer, transfer the top layer along with digestion buffer to a 15ml polystyrene conical tube with final volume of 10ml using a pipet that has been coated with BSA-PBS solution. Following transfer, pipet up and

down 15 to 20 times to dislodge the organoids by disrupting the fat. Centrifuge the solution at 1500rpm for 15 minutes at RT.

12. Following centrifugation, discard the supernatant and resuspend the pelleted organoids in 10ml of DMEM/F12 medium and combine with the pelleted organoids from the first centrifugation. Centrifuge the resuspended fractions at 1500rpm for 15 minutes at RT.
13. Aspirate the medium and resuspend the resulting pellet in 4ml of DMEM/F12 and add 40µl of DNase I solution. Incubate the organoid suspension in a 37°C water bath with gentle agitation for 5 minutes to degrade contaminating DNA.
14. After DNase I treatment add 6ml of DMEM/F12 medium to the mixture and centrifuge at 1500rpm for 15 minutes.
15. Aspirate the supernatent and resuspend the pellet with 10ml of DMEM/F12 medium. Place the tube in a centrifuge and pulse the sample to 1500rpm then immediately brake. This will separate the organoids from the single cells.
16. Aspirate the supernatant and resuspend the resulting pellet with 10ml of DMEM/F12 medium. Place the tube in a centrifuge and pulse the sample to 1200rpm and immediately brake. This will further enrich for organoids.
17. After the second pulse, aspirate the supernatant and resuspend the pellet with 4ml of DMEM/F12 medium. Determine organoid concentration by pipetting 30ul of the resuspended organoids to a 100mm tissue culture dish and count using an inverted phase contrast microscope.
18. Add 6 ml of DMEM/F12 to the tube and centrifuge at 1200rpm for 1 minute at RT.
19. Aspirate the supernatant and resuspend the organoids to a concentration of 10 organoids/µl with ITS/PS medium (defined above) and keep organoids on ice until they are embedded in a 3D matrix, depicted in Figure 3.2

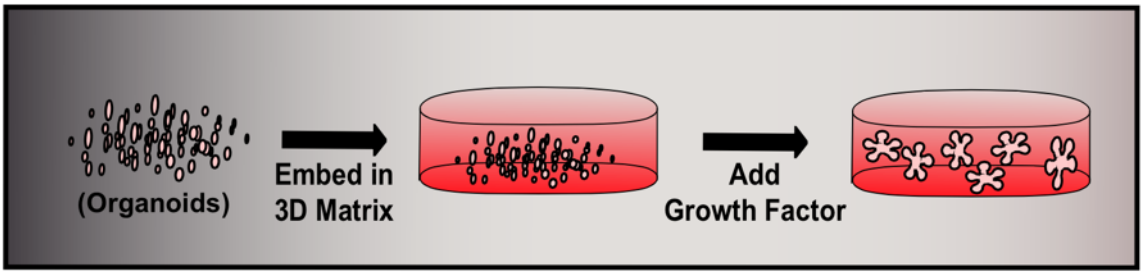

Figure 3.2 Diagrammatic representation of primary mouse organoids embedded in a three-dimensional matrix culture

3.2.3 – Methodology for embedding primary mouse organoids in Matrigel™ for the study of alveolargenesis

The number of organoids placed in Matrigel™ will vary due to the surface area and volume of the well or chamber. For our studies we use a 48-well plate for organoids cultured in Matrigel™ and found a concentration of 200 organoids per well to be optimum.

1. After primary organoid isolation, transfer 400 organoids to a BSA-PBS coated 1.5ml Eppendorff tube and centrifuge at 1500rpm for 1 minute.
2. Aspirate the supernatant and place the tube on ice.
3. Matrigel™ is always kept on ice to prevent premature gelling of the matrix. Pipet 80µl of Matrigel™ into two wells in a 48-well plate and use a P-200 pipet tip to evenly spread the gel to coat the wells evenly.
4. After coating the wells with Matrigel™ place the 48-well plate into a humidified incubator at 37°C with 5% CO_2 for 5 minutes to solidify the matrix.
5. Working quickly, resuspend the organoid pellet on ice with 400µl of Matrigel™. Remove the 48-well plate and pipet 200µl of the organoid suspension into each well and return the plate to the humidified incubator for 5 minutes to solidify the gel.
6. Remove the plate from the incubator and add 2 drops of Matrigel™ with a P-1000 pipet (i.e. approximately 100µl) to each well to fully encase the organoids with Matrigel™. Return the plate to the humidified incubator for 5 minutes to solidify the top gel coat.
7. Remove the plate from the incubator and add 400µl ITS/PS (described above) to each well and return the culture to the humidified incubator.
8. After 24 hours, change the medium with ITS/PS and add TGFα to a single well at a final concentration at 9nM. TGF✔ or other growth factors are required to induce alveolargenesis (Fata et al. 2007). One well is left without growth factor to serve as a negative control.
9. Media is changed every 2 days to 3 days and the cultures are harvested at 6 days after the initial embedding, depicted in Figure 3.3 is a time course image of the organoids for 90 hours.

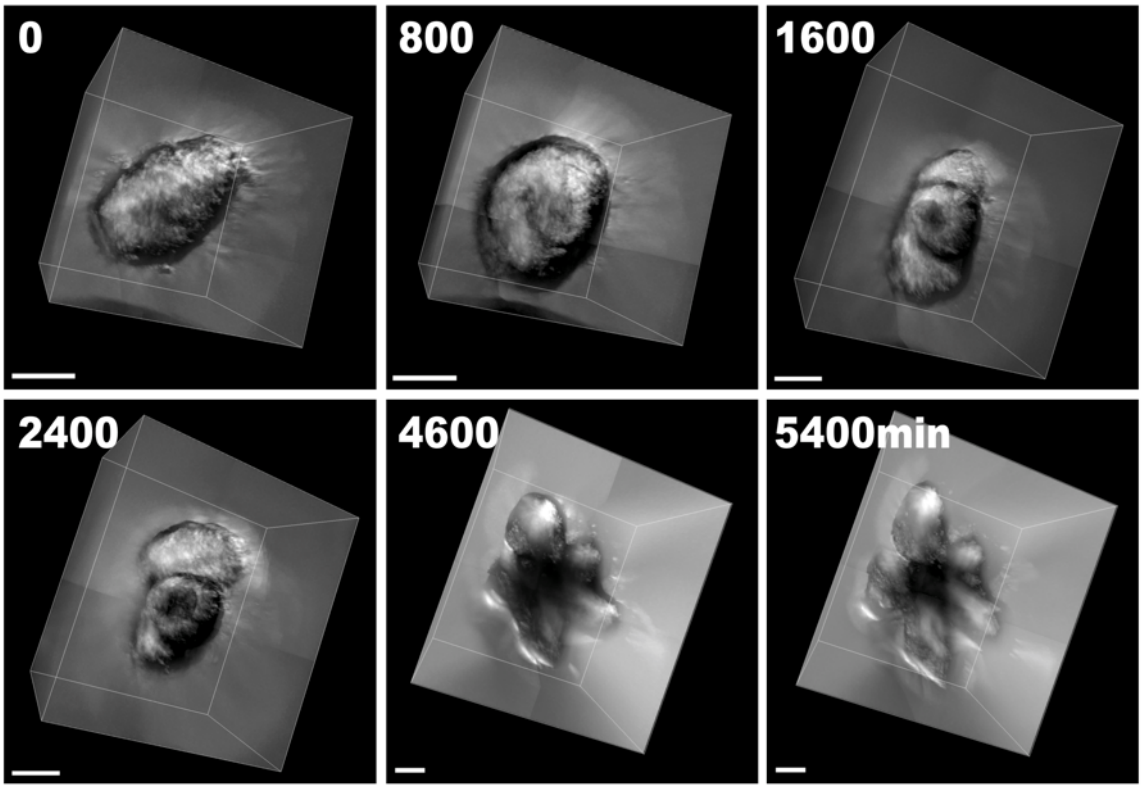

Figure 3.3 Primary mouse organoid culture in laminin-rich gels in response to 9nM TGFα imaged with a timed phase contrast microscopy showing the development process of alveolargenesis beginning when TGFα is added to the culture at time 0. Scale bars are at 50μm.

3.2.4 – Methodology for embedding primary mouse organoids in collagen-1 gels for the study of ductal invasion and elongation

Placing organoids in a collagen-1 gel is slightly different from method described above involving Matrigel™. Due to the collagen extraction process involving acid solubilization, polymerization of the collagen fibers by increasing the pH is required for gelation. The number of organoids embedded in collagen will also vary due to the surface area and volume of the well or chamber. For our studies we used an 8-well borosilicate chamber slide for organoid culture in collagen-1 and found a concentration of 200 organoids per well to be optimum.

1. After primary organoid isolation, transfer 400 organoids to a BSA-PBS coated 1.5ml Eppendorff tube and centrifuge at 1500rpm for 1 minute.
2. Aspirate the supernatant and place the tube on ice.
3. Working quickly, keep the collagen-1 matrix on ice and transfer 1.6ml of collagen-1 into a 50ml conical tube on ice.

4. Polymerize the collagen-1 matrix by adding 200µl 10X DMEM/F12 solution and 200µl 0.1N NaOH and mix by pipetting up and down all while keeping the solution on ice.
5. Dilute the collagen-1 matrix with ITS/PS medium to a final concentration of 3mg/ml.
6. Add 80µl of 3mg/ml collagen-1 solution to two wells in an 8-well borosilicate chamber slide and use a P-200 pipet tip to evenly spread the gel.
7. Incubate the chamber slides in a humidified incubator at 37°C with 5% CO_2 for 5 minutes to solidify the collagen-1 matrix.
8. Resuspend the organoid pellet with 400µl of 3mg/ml collagen-1 solution on ice. Remove the chamber slide from the incubator and add 200µl of the resuspended organoids to each well. Return the chamber slide to the humidified incubator for 5 minutes to solidify the collagen/organoid mixture.
9. After solidifying the collagen-1 gel remove the chamber slide from the incubator and add 2 drops of the 3mg/ml collagen solution with a P-1000 pipet (approximately 100µl) to each well to fully encase the organoids in collagen-1. Return the chamber slide back to the humidified incubator for 5 minutes to solidify the matrix.
10. Remove the chamber slide from the incubator and add 400µl of ITS/PS with or without 9nM of TGFα. The well without TGFα will serve as a negative control.
11. Media was changed every 2 to 3 days and the cultures were harvested after 5 days after initial embedding, depicted in Figure 3.4 is a time course image of the organoids for 25 hours.

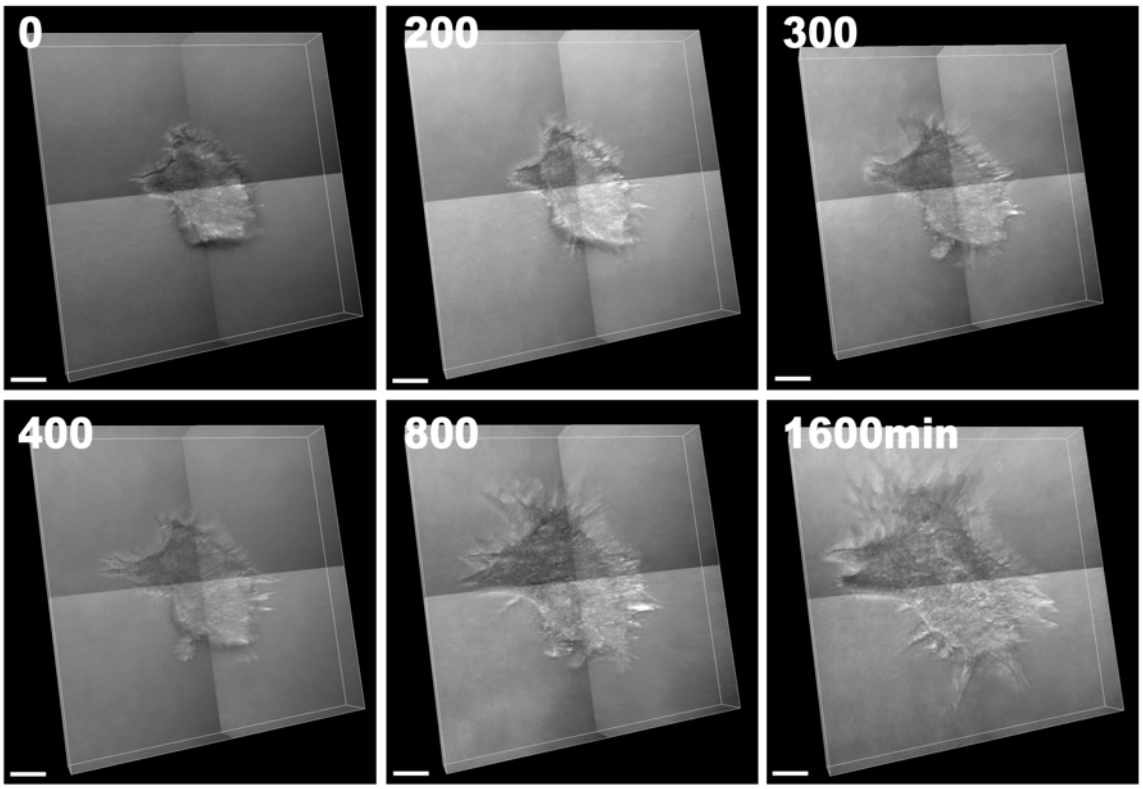

Figure 3.4 Primary mouse organoid culture in Collagen-1 gels in response to 9nM TGFα imaged using timed phase contrast microscopy over 26 hours illustrates the branching morphogenesis program. Scale bars are at 50μm.

3.2.5 – Methodology for immunofluorescence staining of embedded primary mouse organoids in Matrigel™

1. After 6 days in culture begin by aspirating the medium from each well and add 400μl of 18% (w/v) sucrose in PBS to each well and incubate at room temperature on a rotator at 25rpm for 15 minutes. This will help preserve the organoid structure.
2. Exchange the 18% (w/v) sucrose with 30% (w/v) sucrose in PBS and incubate at room temperature on a rotator at 25rpm for 15 minutes.
3. Aspirate away the 30% (w/v) sucrose and pipet up and down with a P-200 pipet to break apart the 3D culture and transfer the mixture and smear it on to a glass slide.
4. Place the glass slide on a 37°C heat block for 1 hour to allow the Matrigel™ and organoid mixture to dry.
5. Prepare a 1:1 Methanol/Acetone mixture fixative. Place slides in a coplin jar with the fixative and incubate for 20 minutes at -20°C.
6. Remove the slides from the fixative and place them on kimwipes for 5 minutes at room temperature to dry.
7. Place slides into a coplin jar with permeablization buffer and incubate at room temperature for 30 minutes.
8. Remove slides from the permeablization buffer and place them in a coplin jar with blocking buffer at room temperature for 1 hour.

9. Remove slides from the coplin jar and place them in 100mm dishes.
10. Dilute primary antibody in blocking buffer at manufacturer's recommended dilution. Drip 500µl of the diluted antibody onto each of the organoid smears and place a piece of parafilm on top to prevent the solution from drying.
11. Place the 100mm dish into a humidified container and incubate the primary antibody solution at 4°C overnight.
12. After overnight incubation, remove the parafilm and place the slides in a coplin jar containing PBS and incubate at room temperature for 15 minutes. Repeat this step 3 times to wash away non-specific binding of the antibody.
13. Remove the slides from the coplin jar and place them in 100mm dishes.
14. Dilute secondary antibody in blocking buffer at manufacturer's recommended dilution. Drip 500µl of the diluted antibody onto each of the organoid smears and place a piece of parafilm on top to prevent the solution from drying.
15. Place the 100mm dish into a humidified container shielded from light and incubate the secondary antibody solution at room tempature for 1 hour.
16. After secondary antibody incubation, remove the parafilm and place the slides in a coplin jar containing PBS and incubate at room temperature for 15 minutes. Repeat this step 3 times to wash away non-specific binding of the antibody.
17. Remove the slides from the coplin jar and place them in a 100mm dish.
18. Drip 500µl of DAPI solution onto the organoid smears and place a piece of parafilm on top to prevent the solution from drying.
19. Place the 100mm dish into a humidified container shielded from light and incubate the DAPI solution at room temperature for 20 minutes.
20. After DAPI incubation remove the parafilm and place the slides in a coplin jar containing PBS and incubate at room temperature for 15 minutes. Repeat this step 2 times to wash away excess DAPI.
21. Remove the slides from the coplin jar and gently dab off the excess PBS and place them 100mm dishes.
22. Add 300µl of Fluoromount-G on top of the organoid smears and place a 1.0 coverslip on top.
23. Place the 100mm dishes in a box shielded from light and let the slides dry overnight before imaging on a scanning confocal microscope, depicted in Figure 3.5 are organoids treated with or without growth factors stained with actin and DAPI.

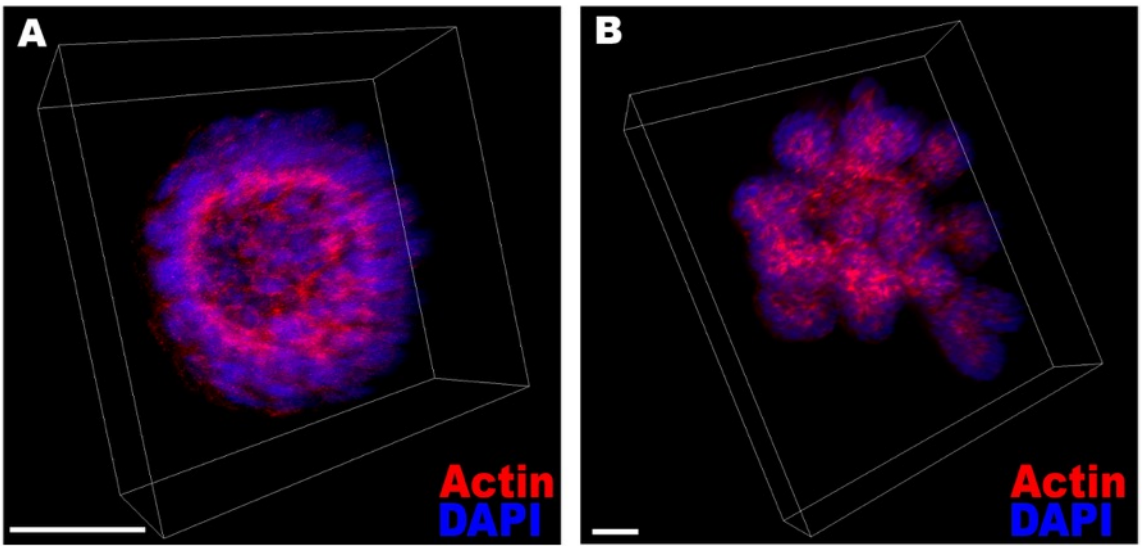

Figure 3.5 Immunofluorescent staining of primary mouse organoids cultured in laminin-rich (lrECM) gels. (A) Untreated organoids grown in lrECM for 5-days stained with phalloidin conjugated to Alexa Fluor 594 and counterstained with DAPI to observe the actin cytoskeleton. (B) 9nM TGFα treated organoids grown in lrECM for 5-days stained with phalloidin conjugated to Alexa Fluor 594 and counterstained with DAPI illustrate a distinct cytoskeleton organization compared to the untreated sample. Scale bars are at 30μm.

3.2.6 – Methodology for immunofluorescence staining of embedded primary mouse organoids in collagen-1 gels

1. After 5 days in culture begin by aspirating the media from each well and add 400μl of 4% (w/v) PFA in PBS to each well and incubate at room temperature for 20 minutes to fix the culture.
2. Aspirate the 4% PFA from each well and replace 400μl of 2% (w/v) glycine in PBS and incubate at room temperature for 15 minutes to quench the PFA fixation.
3. Remove the glycine solution and add 400μl of permeablization buffer and incubate for 1 hour to permeablize the gel.
4. Aspirate the permeablization buffer and replace with blocking buffer. Incubate at room temperature for 1 hour.
5. Dilute primary antibody in blocking buffer at manufacturer's recommended dilution. Aspirate blocking buffer and add 250μl of the diluted antibody to each well and place the chamber slide in a humidified container.
6. Incubate the primary antibody solution at overnight at 4°C.
7. After incubation aspirate the primary antibody solution from each well and replace with 300μl of PBS and incubate at room temperature for 15 minutes. Repeat this step 3 times to wash away non-specific antibody binding.
8. Dilute secondary antibody in blocking buffer at manufacturer's recommended dilution. Aspirate PBS and add 250μl of the diluted antibody to each well and place the chamber slide in a humidified container shielded from light.

9. Incubate for 1 hour at room temperature.
10. Aspirate the secondary antibody solution and replace with 300µl of PBS and incubate in a container shielded from light at room temperature for 15 minutes. Repeat this step 3 times to wash away non-specific antibody binding.
11. Aspirate PBS from each well and replace with 250µl of DAPI solution. Incubate chamber slide at room temperature shielded from light for 20 minutes.
12. Aspirate DAPI solution from each well and add 300µl of PBS and incubate at room temperature shield from light for 15 minutes. Repeat the step 2 times to wash away excess DAPI.
13. Image on a scanning confocal microscope, depicted in Figure 3.6 are organoids treated with or without growth factors stained with actin and DAPI.

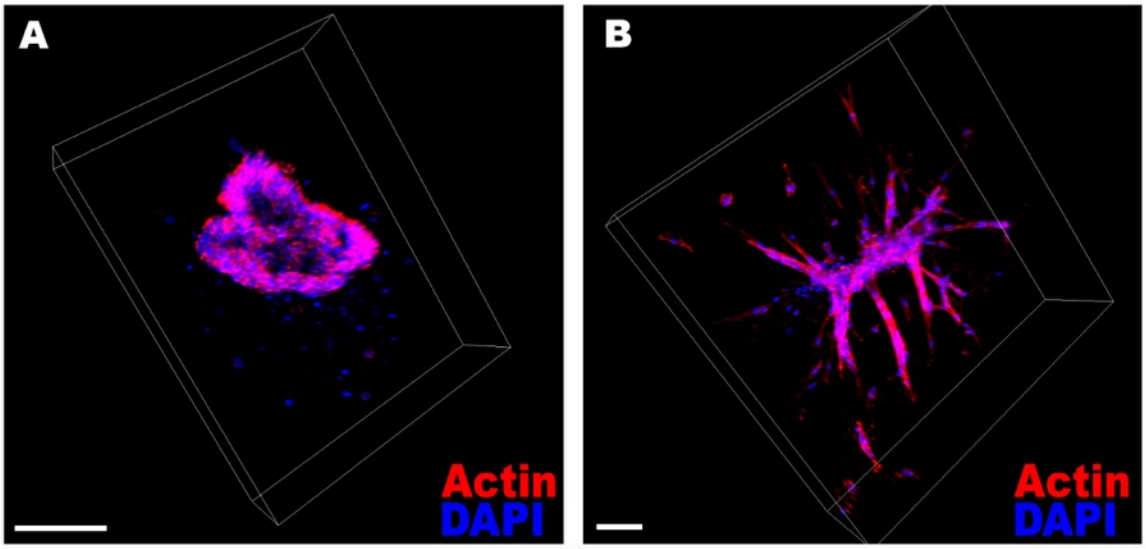

Figure 3.6 Immunofluorescent staining of primary mouse organoids cultured in collagen-1 gels. (A) Untreated organoids grown in collagen-1 for 4-days stained with phalloidin conjugated to Alexa Fluor 594 and counterstained with DAPI to observe the actin cytoskeleton. (B) 9nM TGFα treated organoids grown in collagen-1 for 4-days stained with phalloidin conjugated to Alexa Fluor 594 and counterstained with DAPI illustrate a distinct cytoskeleton organization compared to the untreated sample. Scale bars are at 100µm.

3.3 – Discussion

Here we describe a generalize protocol for the isolation and culture of primary mouse mammary organoids in 3D gels illustrating the disparate phenotypes observed as a result of the different matrix components in which they are cultured. We can outline distinct morphogenic processes observed in the mouse mammary gland. For instance, culturing primary organoids in Matrigel™ will allow organoids to undergo the developmental program reminiscent of alveologenesis when properly induced by growth factors. This is in rather stark contrast to the branching morphogenesis phenotype observed when cultured in collagen-1 gels. Using these approaches, we hope to further develop models

that will incorporate other cell types to more appropriately represent the microenvironment in an ex vivo culture system.

Chapter 4 – The involvement of the non-catalytic domain of matrix-metalloproteinase 14 in branching morphogenesis

4.1 – Introduction

The mammary gland is an organ that is remodeled and expanded by ductal invasion during branching morphogenesis (Sekhri et al. 1967; Hogg et al. 1983). Ductal invasion occurs primarily during puberty and is driven by the terminal end bud bifurcation and lateral branching generating an epithelial tree-link structure (Neville & Daniel 1987; Fata et al. 2004). Concomitant with this process, remodeling of the gland happens and an increase of matrix metalloproteinases (MMPs) occurs. These MMPs constitute a family of degradative enzymes that are responsible for the physiological and pathological changes extracellular matrix changes within the mammary gland (Talhouk et al. 1991; Sympson et al. 1994; Witty et al. 1995; Fata et al. 1999). Studies using engineered mice and organotypic culture models have demonstrated that regulated MMP proteolytic activity is required for normal mammary gland function (Simian et al. 2001; Wiseman et al. 2003). For example, delayed terminal end bud invasion at day 30 post partum was observed in Mmp2 knockout mice, but by day 50 the branching caught up to that of the normal gland (Wiseman et al. 2003). Conversely, studies investigating the over-expression of MMP3, 7, and 14 found that mice up-regulating these proteins have cause ductal hyperplasia (Sympson et al. 1994; Witty et al. 1995; Rudolph-Owen et al. 1998; Sternlicht et al. 1999; Ha et al. 2001). Interestingly, using in-situ hybridization, MMP14 was shown to be expressed around the invading front of the terminal end buds (Wiseman et al. 2003), which suggests that it may play a role in mammary epithelial invasion into the fat pad. Although studies such as these are very informative, the mechanism in which the signaling events involved in controlled physiological invasion through the fibrous stroma during development of the normal epithelial cells is still not well understood. Confounding the understanding of the process is the observation that the density of fibrillar collagens changes in a context depended manner during mammary gland development caused by the epithelial cells invading into the fat pad. During puberty, these dense bands of collagen fibers are present as a sheath in the periductal stroma surrounding the subtending ducts but are absent in the stromal regions at the tips of the invading end buds (Silberstein et al. 1990; Keely et al. 1995; Ingman et al. 2006). This suggests that stromal collagen may act as a mechanical barrier to prevent invasion and that collagen remodeling would be required for end bud penetration into the stroma. Reducing tissue rigidity by increased MMP activity (Simian et al. 2001) or by pharmacological inhibition of cellular contractility (Moore et al. 2005) has been shown to increase epithelial branching. However, earlier studies have shown a requirement also for collagen synthesis and deposition during branching morphogenesis (Spooner & Faubion 1980; Fukuda et al. 1988). Any underlying mechanisms by which collagen and MMPs coordinate mammary ductal invasion have yet to be fully explored. This may be due to the fact that detailed analysis of morphogenetic invasion *in vivo* is technically challenging because the mammary gland changes rapidly and continuously and crucial pathways may sometimes be reciprocally activated or suppressed. Furthermore, the mammary gland is comprised of distinct cell types constantly interacting with each other,

as well as a variable mix of ECM molecules, hormones, growth factors and proteinases, all of which make it difficult to isolate a singular pathway in time and space to study, even with the use of engineered animals.

To examine the relative roles of each distinct entity and yet remain in a physiological context, we used mammary epithelial cell lines and primary mammary epithelial organoids grown in a three-dimensional (3D) system to decipher the role of MMP14 in branching morphogenesis. This experimental strategy allowed us to tune MMP14 expression as well as the local density of the 3D matrix. Using this model, we were able to uncover many dynamic and reciprocal pathways required for mammary branching morphogenesis. We found that collagen density controls the level of MMP14 expression and that although proteolytic activity of MMP14 could be dispensed with, in some conditions the presence of MMP14, in particular the non-catalytic domains are essential for branching. Furthermore, we show that MMP14 dependent branching requires β1-integrin and that MMP14 expression influences the expression of β1-integrin and affects the integrin function. Lastly, we identify the intracellular signaling mechanism essential for branching to occur in our system. We find that downstream signaling of MMP14 is through activation of Erk1/2 and Src; these signaling nodes drive epithelial invasion and allow branching and that activation of MAPK signaling can rescue the branching, cell adhesion, and traction in a MMP14 independent manner. Given that high level of MMP14 expression in breast cancer, we propose that these findings identified during normal branching epithelium have relevance also to the role of stromal density in breast cancer risk.

4.2 – Methods

Cell culture and reagents

Functionally normal mouse mammary epithelial cells, EpH4, were cultured in 1:1 Dulbecco's Modified Eagle's Medium: Ham's F12 Nutrient Mixture (DMEM/F12) with 2% fetal bovine serum (Clonetech), 5μg/ml insulin (Sigma), 50μg/ml gentamycin (Sigma). The following inhibitors were used at the concentrations indicated: PD98059 (40μM; Calbiochem); GM6001 (40μM; Chemicon); PP2 and inactive analogue, PP3 (10μM; Calbiochem).

Preparation of lenitvirus for transduction

Lentivirus was generated using modified viral construct backbones generated by Eric Campeau at the Lawrence Berkeley National Laboratory. Lentiviral plasmids were transfected into 293FT cells (Invitrogen) using Fugene 6 (Roche) a lipid based transfection agent. Transfected cells were cultured in DMEM media containing 10% FBS, 0.1 mM MEM Non-Essential Amino Acids (Invitrogen), 6mM L-glutamine (Invitrogen), 1mM MEM Sodium Pyruvate (Invitrogen) and 500μg/ml Geneticin (Invitrogen). After initial transfection, the media was replaced after 24 hours with fresh media and then incubated for 48 hours before the media was collected. After the media was collected, the virus was concentrated by ultracentrifugation at 100,000g using a SW41Ti rotor (Beckman) for 90

minutes. The concentrated virus was either used to transduce the appropriate cells or frozen at -80°C until needed.

Preparation of collagen-I from rat tails

Rat tails were obtained from the Jackson Laboratory (Bar Harbor, ME) at 12 weeks of age. The rat tails were incubated in 70% ethanol for 12 hours for disinfection. After disinfection, the tails were skinned to expose the underlying tendons were the collagen-I will ultimately be extracted from. Using surgical tools, the tendons embedded within the muscles of the tails were extracted and cleaned. After extraction, the tendons were incubated in 1.0N acetic acid for 24 hours. After acid extraction, the collagen-I was clarified using ultra-centrifugation at 25,000rpm for 1 hour. After centrifugation, the collagen-I supernatant was retained and dialyzed in 0.01N HCl over 72 hours. After dialysis, the solution was then ready for use.

Preparation of collagen-I gels for three-dimensional culture

Acid-soluble collagen-I was obtained either prepared from rat tails (Jackson Labs) or purchased commercially (BD Bioscience, Koken) and then is neutralized by addition of 10X DMEM/F12 1:1 solution (1 volume), 0.1N NaOH (1 volume), and the acid-soluble collagen-I (8 volumes) on ice. After neutralization, the collagen-I mixture is then diluted to a final concentration of 3mg/ml using media consisting of DMEM/F12 1:1 supplemented with 10µg/ml insulin, 5.5µg/ml transferrin, and 1ng/ml sodium selenite (ITS supplement, Sigma) and 100U/ml penicillin-streptomycin (Sigma). After generating the final concentration of the collagen-I mixture, it is incubated on ice for 30 minutes before use.

Branching morphogenesis assay

Branching morphogenesis assays was done in three-dimensional collagen-I gels. Preparation of collagen-I gels was done according to the method described above. EpH4 cells were cultured for 48 hours in suspension on polyhema coated 6-well plates to generate EpH4-culsters. After clustering, the cells were spun down by pulse centrifugation at 1,000rpm to retain only large clusters. The clusters were then pelleted and resuspended in the pre-prepared collagen-I matrix. Within a well in a 48-well plate, a layer of collagen-I is pipetted to generate a thin coating. Afterwards, 200 cell clusters within the collagen-I matrix is layered on top at a concentration of 200 clusters per 200µl collagen-I. After layering the cells in the well, the plate is incubated at 37°C for 5 minutes. After incubation, another 100µl of collagen-I is added to the top and the plate is then incubated again for 5 minutes at 37°C. After this incubation, 400µl of media is added to the well with or without growth factors and chemical inhibitors. The assay will continue for 6 days with culture media changes every 2 days.

Whole mount β-galactosidase staining

Transgenic mice carrying the *LacZ* gene under the control of the MMP14 promoter were used (Yana et al. 2007). Inguinal mammary glands were isolated from 12 week old female wild-type (+/+) and MMP14 (+/-, *lacZ*) mice. Mammary glands were processed in ice-cold PBS and then incubated for 15 minutes at room temperature in a fixative solution consisting of 2% formaldehyde, 0.2% glutaraldehyde, 0.02% Nonidet P-40 and 0.01% sodium deoxycholate in PBS. After fixation, glands were rinsed several times in cold PBS before an overnight incubation at 37°C in an enclosed chamber shielded from light submerged in staining solution consisting of 5mM potassium ferricyanide and 5mM potassium ferrocyanide in rinse buffer, 1mg/ml β-Galactosidase, 2mM $MgCl_2$, 0.02% Nonidet P-40 and 0.01% sodium deoxycholate in PBS. After staining, the mammary glands were dehydrated and embedded in paraffin before serial sections were made and stained with Hematoxylin and Eosin.

Gel stiffness probed by AFM

Gels were prepared by adding 100µl of collagen solution either 1mg/ml or 3mg/ml to the glass surface of a 35mm culture dish with a 14mm diameter bottom-glass coverslip (MatTek, Ashland, USA) and incubating the samples for 20 minutes at 37°C to allow gelation. Gel stiffness was characterized by measuring the Young's elastic modulus (*E*) using an atomic force microscope (AFM) (Bioscope; Bruker AXS, Santa Barbara, USA). For measurements of the collagen-1 gel at least nine random gel locations were collected. In brief, force (F) measurements were conducted using low spring constant cantilevers (k = 0.03Nm^1) (Microlever, Veeco). We also measured the loss modulus G^{00} of collagen-1 gels with AFM using low-amplitude (75nm) and low-frequency (o = 20.4Hz) oscillations and calculated the corresponding dynamic viscosity as $Z = G^{00}/o$. All mechanical data was given as mean SE and correspond to at least 2 independent experiments.

Western blotting

Samples were lysed using RIPA buffer with phosphatase and proteinase inhibitors (50mM HEPES pH 7.4., 150mM NaCl, 10% glycerol, 1% Triton X-100, 10mM sodium pyrophosphate containing 1.5mM $MgCl_2$, 1mM EGTA, 1% sodium deoxycholate, 0.25mM Na_3VO_4, 100mM NaF and proteinase inhibitor cocktail set (Calbiochem)). Protein concentration was measured using the BCA Protein Assay kit (Pierce), following the manufacturer's instrucutions. 25µg protein samples were mixed with Laemmli sample buffer and heated at 95°C for 5 minutes. Samples were loaded into a pre-cast 4-20% Tris-glycine polyacrylamide gel (Invitrogen) using the NOVEX system (Invitrogen). Resolved proteins were transferred to nitrocellulose membrane (Whatman) followed by blocking in PBS containing 0.05% Tween-20 with 5% w/v non-fat dry milk for 1-hour at room temperature (RT). Membranes were incubated overnight at 4°C in 5% BSA, 0.1% Tween-20 in PBS containing antibodies that recognizing either phosphorylated ERK1/2, total ERK1/2 (Cell Signaling), total c-Src, phosphorylated Src (pY416) (Cell Signaling), and total MMP14 (Abcam). Primary antibodies were detected with the Pierce SuperSignal

detection kit and signal was captured with the FluorChem 8900 analysis system (Alpha Innotech, San Leandro, CA).

Quantitative RT-PCR analysis

Total RNA was isolated using QIAGEN RNeasy Mini kit (Valencia, CA). 100ng of total RNA was used to synthesize cDNA using SuperScript II First-Strand Synthesis System (Invitrogen). MMP14 was amplified with 5'-GAGATCAAGGCCAATGTTCG and 5'-GTCCAGGGCTCGGCAGAATC primers or with 5'-CATCTTCTTGGTGGCTGTG and 5'-TGACCCTGACTTGCTTCC primers. β1-integrin was amplified with 5'-GGAGATGGGAAACTTGGTGG and 5'-CCCATTCACCCCATTCTTGC primers. As a control for total RNA, RT-PCR for 18S rRNA was performed with 5'-TCGGAACTGAGGCCATGATT and 5'-CCTCCGACTTTCGTTCTTGATT primers. Real-time PCR was performed using the LightCycler System and Fast Start DNA Master SYBR Green I (Roche) following manufacturer's instructions.

4.3 – Results

4.3.1 – MMP14 expression is increased during ductal invasion *in vivo*

Our investigation focused on MMP14, the dominant pericellular collagenase (Chun et al. 2010), because it has been reported that although other membrane-type (MT) MMPs (MMP15, 16 and 17) are expressed in the mammary gland, their expression is quite low compared to MMP14 (Szabova et al. 2005). Indeed, we found this to the case in the strain of mice used for our investigation. RNA extracted from total mammary gland extracts revealed the expression of other MT-MMPs was quite low compared to MMP14 expression. During mammary gland development, the anlaga actively invades into the fat pad of the gland and upon pregnancy the gland undergoes a rapid expansion of epithelial cells in order to obtain the capacity to produce milk, this process is called lactation. After lactation, the gland undergoes another remodeling process called involution, returning the gland back to a pre-pregnancy state. During the lifetime of the female, the mammary gland is involved in a complex process and tremendous remodeling. First, we began to determine the involvement of MMP14 in the various stages of mammary gland development. Quantitative RT-PCR analysis of the mammary gland from virgin mice showed that expression of MMP14 increases through puberty, but it then reduced during pregnancy and essentially absent during lactation, depicted in Figure 4.1.

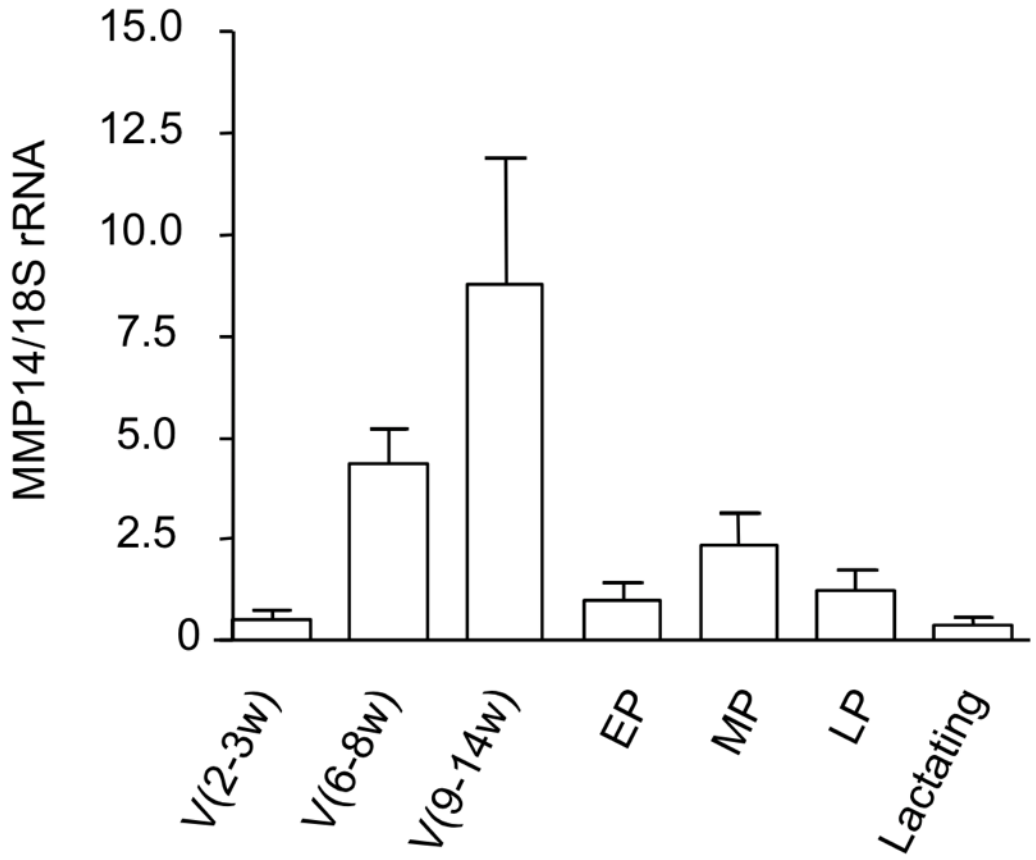

Figure 4.1 Quantitative RT-PCR analysis of MMP14 expression during development of the mouse mammary gland from Virgin (V), early pregnancy (EP), mid pregnancy (MP), late pregnancy (LP), and lactating. The expression was normalized to 18S rRNA and depicts the high levels of MMP14 during late stage mammary development.

To observe the spatial and temporal expression of MMP14 within the mammary gland, we used mice carrying the *LacZ* reporter downstream of the endogenous MMP14 promoter (Yana et al. 2007). B-galactosidase staining of the mammary gland isolated from these mice were used and showed that MMP14 is strongly present at the tips of the ductal tubules and the expression is observed mainly in the ductal epithelium and myoepithelial cells suggesting that expression of MMP14 is spatially located at the interface of the invading epithelium which is essential for branching morphogenesis, depicted in Figure 4.2.

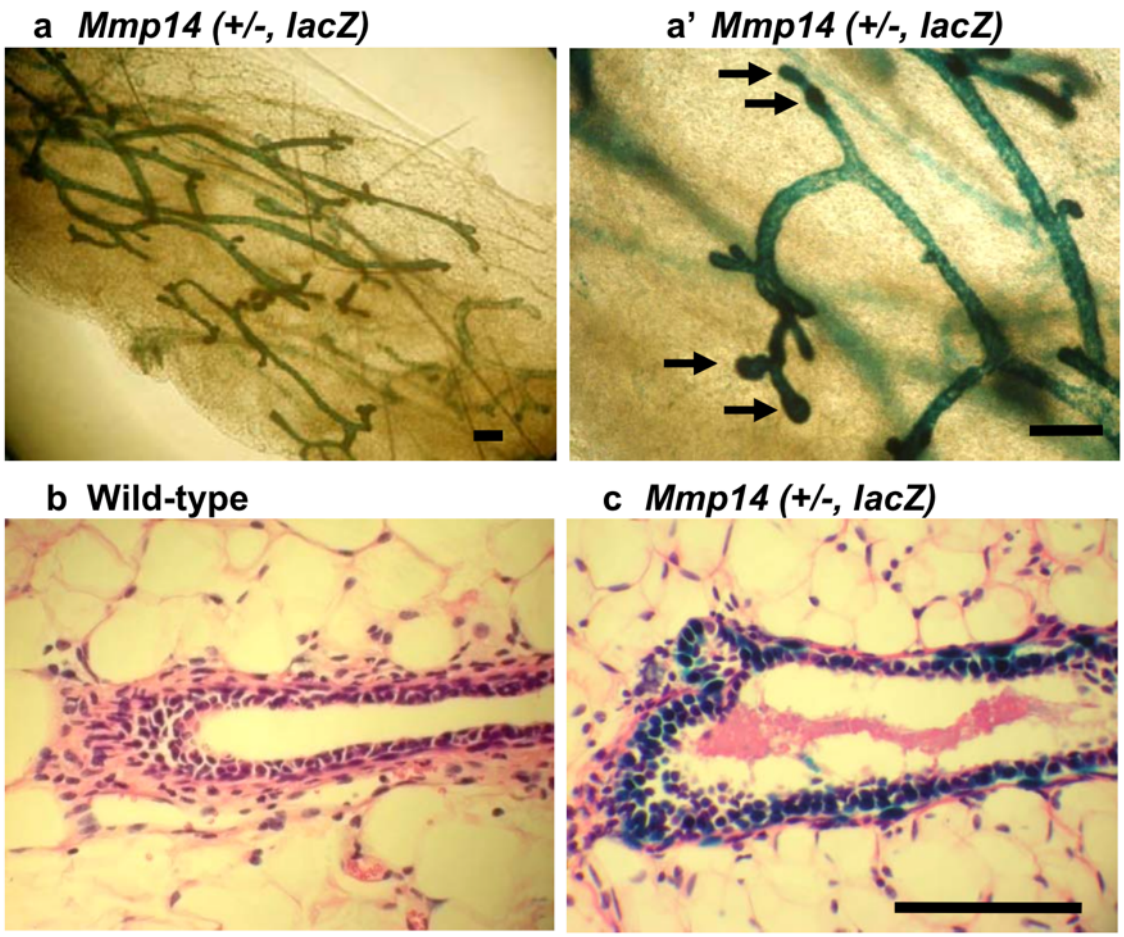

Figure 4.2 Glands from 10 week old mice. (a and a') β-galactosidase staining of a while mount of a mammary gland isolated from virgin transgenic heterozygous mice bearing the lacZ gene under the control of the endogenous MMP14 promoter. (b and c) β-galactosidase along with H&E staining of a mammary gland section from WT mice and MMP14 transgenic mice. Scale bars are at 200μm.

4.3.2 – MMP14 expression is required for branching in 3D culture, and MMP proteolytic activity is required for invasion through dense but not sparse type-I collagen gels.

To elucidate the role of MMP14 in epithelial invasion during branching morphogenesis, we utilized two organotypic culture models to approximate mammary gland branching seen *in vivo*: primary mammary organoids (Simian et al. 2001; Fata et al. 2007) and epithelial clusters (Hirai et al. 1998) of a phenotypically normal mouse mammary epithelial cell line, EpH4 (Reichmann et al. 1989) both embedded in type-I collagen gels. Within a living organism, such as a mouse, the mammary gland epithelium is enveloped by collagen-1, and the stroma itself contained collagen-1, depicted in Figure 4.3.

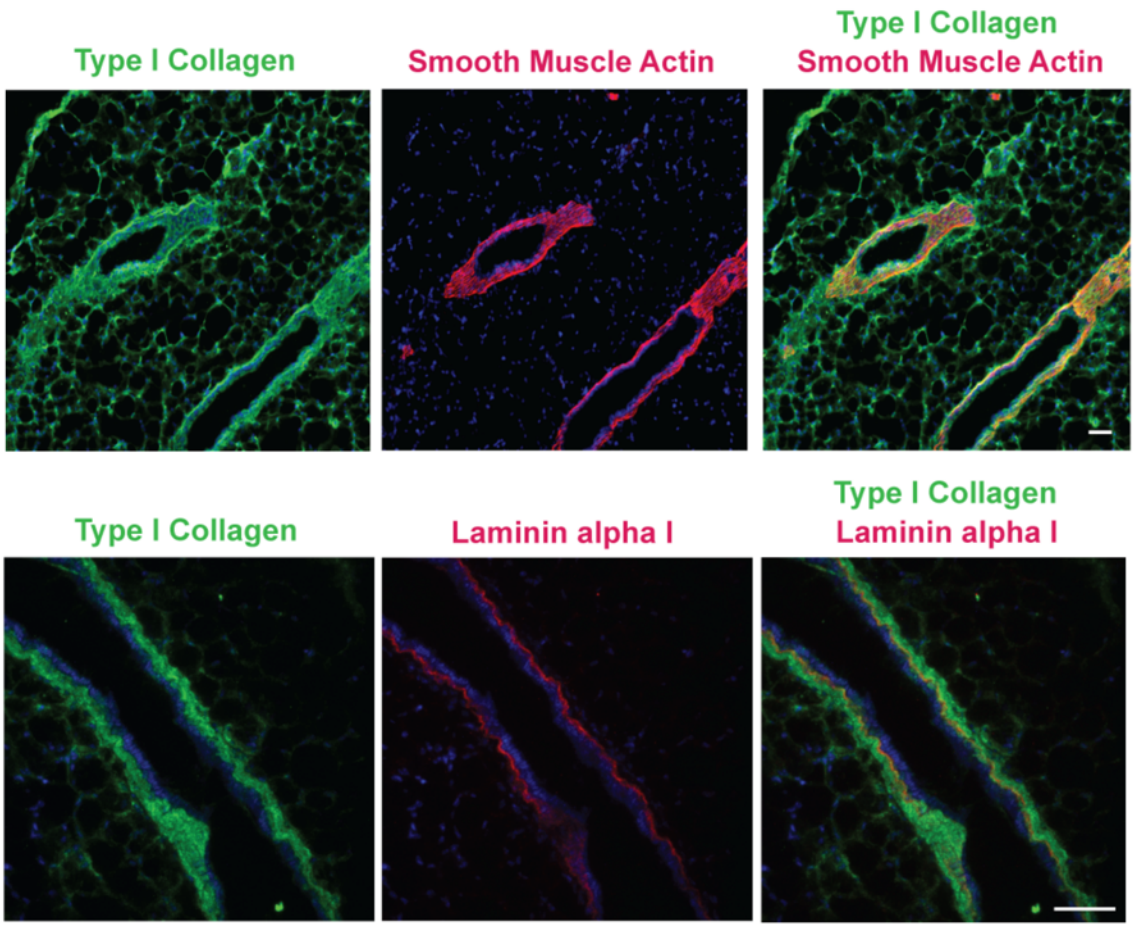

Figure 4.3 Collagen-1 surrounds the ducts of the mammary gland. Immunostaining of 8-week old C57BL/6 mouse mammary gland that were cryosectioned and stained with collagen-1 and smooth muscle actin to depict the myoepithelial layer or laminin alpha I to illustrate the deposited basement membrane.

As a result of the prevalence of collagen-1 within the mammary gland, 3D cultures with collagen-1 is a useful model to investigate branching morphogenesis. Using acid extracted collagen-1 to simulate *in vivo* conditions has been suggested by others and now is used widely to investing mammary branching (Sabeh, Shimizu-Hirota, et al. 2009). Using EpH4 cell clusters we were able to investigate branching morphogenesis in collagen-1 gels ex vivo. Branching was scored as positive when a cluster displayed three or more branched segments of a length at least the diameter of the central cell cluster (Simian et al. 2001). What we discovered was that extensive branching was induced by epidermal growth factor (EGF) or fibroblast growth factor-2 (FGF2) in 3mg/ml collagen, depicted in Figure 4.4.

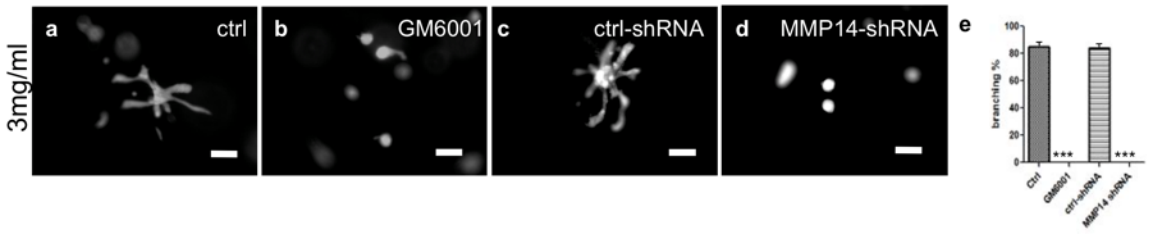

Figure 4.4 EpH4 clusters exhibit branching in 3mg/ml collagen-1 cultures but the process is inhibited when MMP catalytic activity is blocked using GM6001 or when MMP14 is knocked-down using shRNA.

Invasion into the collagen-1 gels was completely abrogated by the addition of GM6001, a broad spectrum MMP inhibitor or by decrease MMP14 expression through shRNA, depicted in Figure 4.4. These data are consistent with previous studies indicating an absolute requirement for MMPs and specifically MMP14 for invasion and branching into collagen-1 gels (Fata et al. 1999; Lee et al. 2001; Simian et al. 2001; Wiseman et al. 2003; Page-McCaw et al. 2007).

MMP14 is a collagenase (Ohuchi et al. 1997), and as depicted in Figure 4.1 is correlated in the mammary gland with periods of increased branching and remodeling. Therefore, we suspected that MMP14 might be required, directly or indirectly for branching, thus we investigated the possibility of reducing the local concentration of collagen to prevent the advancing by the epithelium by means of MMP14. To this end, we asked whether MMP14 was required if the collagen density was reduced. Once we reduced the collagen-1 concentration to 1mg/ml in our assay, we observed that EpH4 cluster were able to formed branched structures. Most importantly, treatment by GM6001 in 1mg/ml collagen-1 cultures did not prevent branching, only down-modulation of the MMP14 gene led to a decrease in branching observed, depicted in Figure 4.5. These data suggest that MMP14 may have a non-catalytic function. MMP14 domains other than the catalytic one are known to have function (Itoh et al. 2001; Mori et al. 2002; Cao et al. 2004).

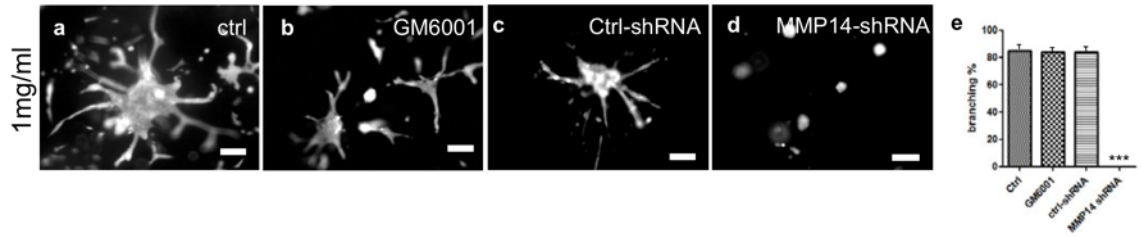

Figure 4.5 EpH4 clusters exhibit branching in 1mg/ml collagen-1 cultures and the process is uninhibited when MMP catalytic activity is blocked using GM6001 but when MMP14 is knocked-down using shRNA the branching phenotype is inhibited.

The observation that inhibition of metalloproteinase activity did not inhibit branching in 1mg/ml collagen-1 gels, but shRNA to MMP14 did suggests that other properties of MMP14 are necessary besides its catalytic ability. To test the requirement of MMP14 non-proteolytic activity during branching we utilized the over-expression of a MMP14 mutant

protein with a deleted catalytic domain (MMP14-dCAT) which was previously shown to act as a dominant negative to MMP14 catalytic activity in fibrosarcoma cells to inhibit invasion and metastasis (Itoh et al. 2001; Nonaka et al. 2005). A catalytic domain inactive mutant was also used (MMP14-E/A) which has a mutation at the Glu-240 residue of the active site for proteolysis (Rozanov et al. 2001). By overexpressing these mutants in MMP14-shRNA treated cells, we were able to dissect out whether MMP14 displays a non-catalytic function during branching morphogenesis, depicted in Figure 4.6. As expected, the branching phenotype of the MMP14-shRNA cells was rescued by over-expression of active MMP14. However, more intriguing was the observation that the branching phenotype was rescued by over expressing MMP14-dCAT or MMP14-E/A, indicating that the MMP14 protein and not the proteolytic activity of MP14 is sufficient for supporting branching in the 1mg/ml collagen-1 gel. These observations suggest that mammary epithelial cells utilize different MMP14 dependent mechanisms to respond to collagen concentration precisely, and use different modes of cellular invasion through different microenvironments.

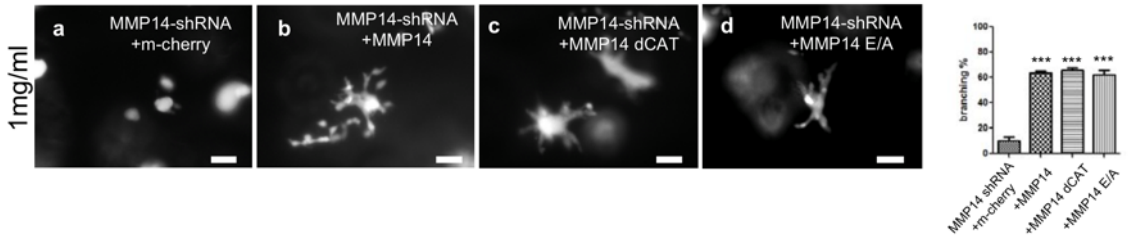

Figure 4.6 EpH4 clusters exhibit branching in 1mg/ml collagen-1 cultures and the process is uninhibited when MMP catalytic activity is blocked using GM6001 but when MMP14 is knocked-down using shRNA the branching phenotype is inhibited.

4.3.3 – MAP kinase drives invasion of mammary epithelial cells through collagen-1 gels

In the previous section we made the observation that catalytic activity of MMP14 was dispensable but the MMP14 protein itself was not during invasion through 1mg/ml collagen-1 gels, which we call sparse collagen-1. We suggested that the non-proteolytic activity of MMP14 were possibly involved in novel signaling mechanisms that allowed for the invasion through the less dense collagen-1 matrix. It has been shown previously the importance of MAP kinase signaling for organoid alveologenesis in a laminin-rich ECM assay (Fata et al. 2007). As such, we hypothesized that the non-proteolytic portion of MMP14 may be involved in influencing MAP kinase signaling during branching morphogenesis.

To identify whether MAP kinase signaling is necessary for ductal branching in collagen, we disrupted MAP kinase activation using MEK inhibitor, PD98059 (Dudley et al. 1995). Blocking MAP kinase activity prevented branching in both 3mg/ml and 1mg/ml collagen-1 gels, depicted in Figure 4.7. However, inhibitors preventing the activity of PI3K and NF-κB had no effect on branching in 1mg/ml sparse collagen-1 gels, and PI3K inhibitors reduced branching only slightly in 3mg/ml dense collagen. Thus MAP kinase activity is

essential for mammary cell branching and elongation in both 3mg/ml and 1mg/ml collagen concentrations.

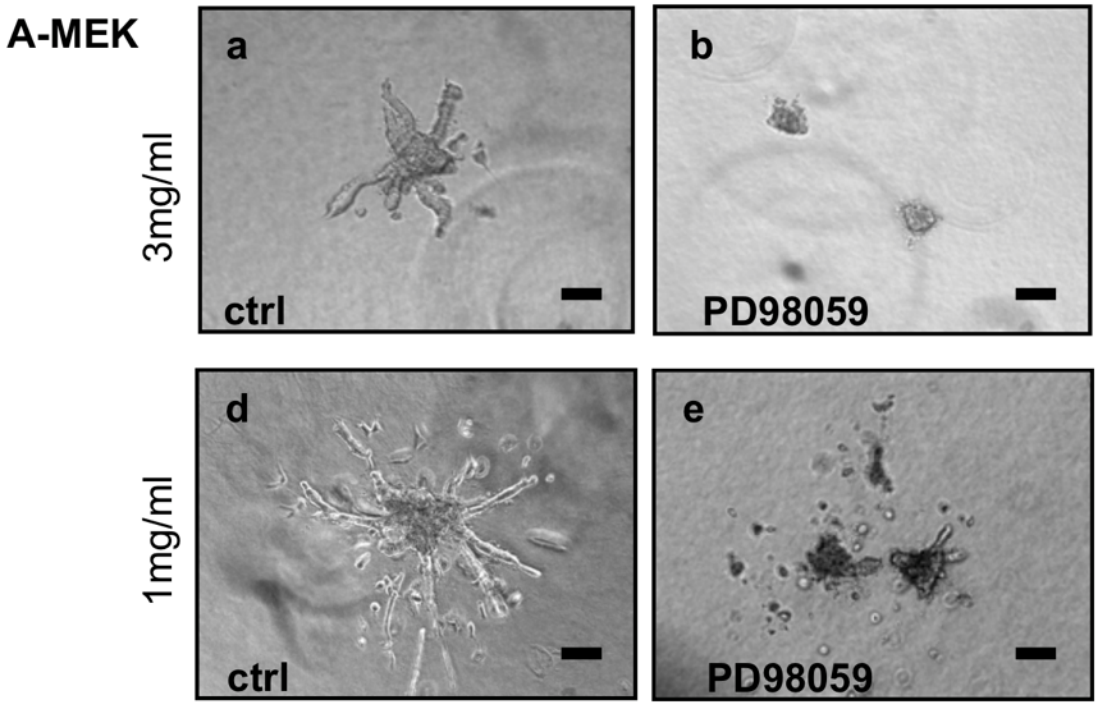

Figure 4.7 EpH4 clusters exhibit branching in 3mg/ml and 1mg/ml collagen-1 gels treated either with DMSO (ctrl) or a MEK inhibitor (PD98059)

Even though these data illustrate MAP kinase signaling is essential for branching morphogenesis, it was possible that other signaling modules were also involved. It was previously shown that c-Src null mice have reduced mammary gland branching (Kim et al. 2005). Because this knockout was not tissue specific, we tested whether Src is involved in mammary epithelial branching. The presence of the Src inhibitor, PP2 (Hanke et al. 1996) prevented branching in both 3mg/ml and 1mg/ml collagen-1 gels, depicted in Figure 4.8. Additionally, PP2 inhibited activation of Erk, this suggests that Src is an upstream regulator for Erk activation in these conditions. Thus Src activity is also essential for mammary cell branching in collagen.

Src activity has shown to be controlled by ligation between integrins and ECM, and integrins have been reported to be involved in human mammary epithelial branching morphogenesis in collagen gels (Calalb et al. 1995; Klinghoffer et al. 1999; Hynes 2002; Berdichevsky et al. 1994). To determine whether reduction of integrin signaling affects branching morphogenesis of EpH4 clusters, β1-integrin shRNA treated cells were tested in the collagen-1 branching assay. The knockdown of β1-integrin by shRNA stopped branching in both 3mg/ml and 1mg/ml collagen-1 gels and showed the same morphology as inhibition by MEK or Src using inhibitors, depicted in Figure 4.9. These results suggest

there is a β1-integrin dependent Src/Erk activation that is necessary for branching morphogenesis in mammary epithelial cells in a collagen-1 matrix.

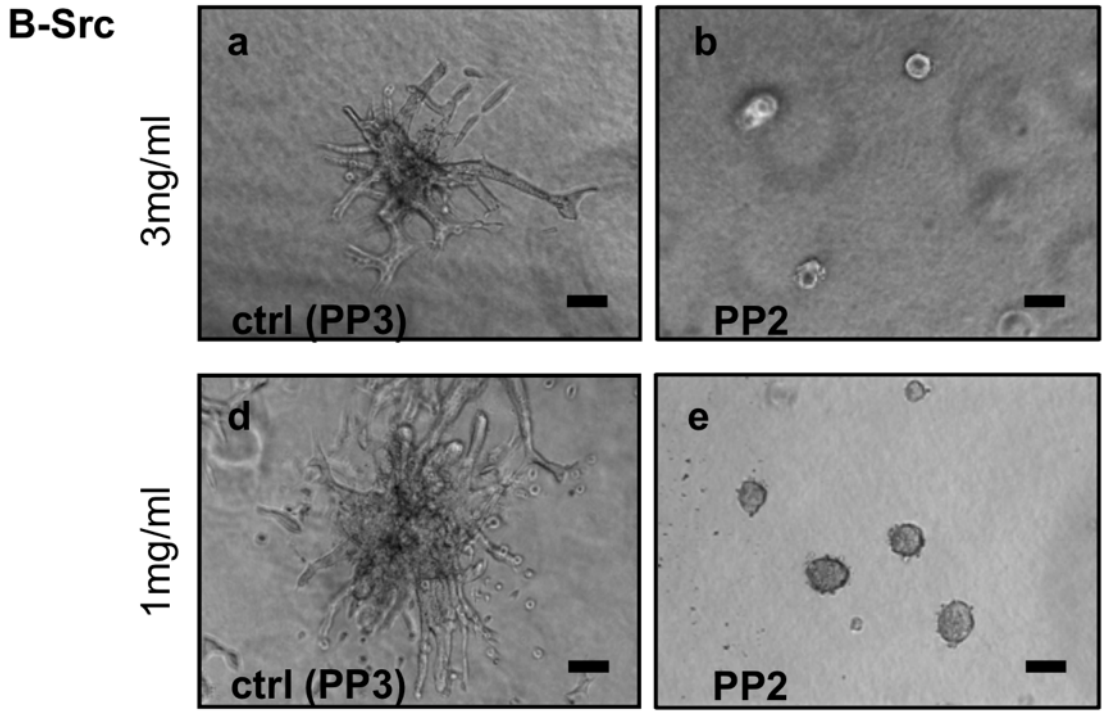

Figure 4.8 EpH4 clusters exhibit branching in 3mg/ml and 1mg/ml collagen-1 gels treated either with PP3 and negative control for the PP2 (ctrl) or an Src inhibitor (PP2)

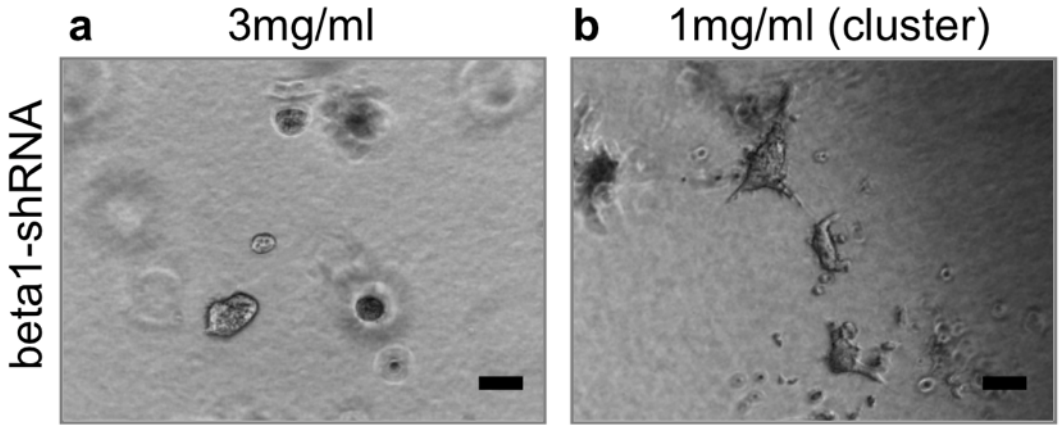

Figure 4.9 EpH4 clusters treated with β1-integrin shRNA do not exhibit branching in both 3mg/ml and 1mg/ml collagen-1 gels

4.3.4 – MMP14 and β1-integrin control Erk activation in EpH4 cells in collagen

It has been shown that over-expression of MMP14 is able to activate Erk via cell-ECM association in African green monkey fibroblast cells and in osteosarcoma cells (Gingras et al. 2001; Takino et al. 2004).

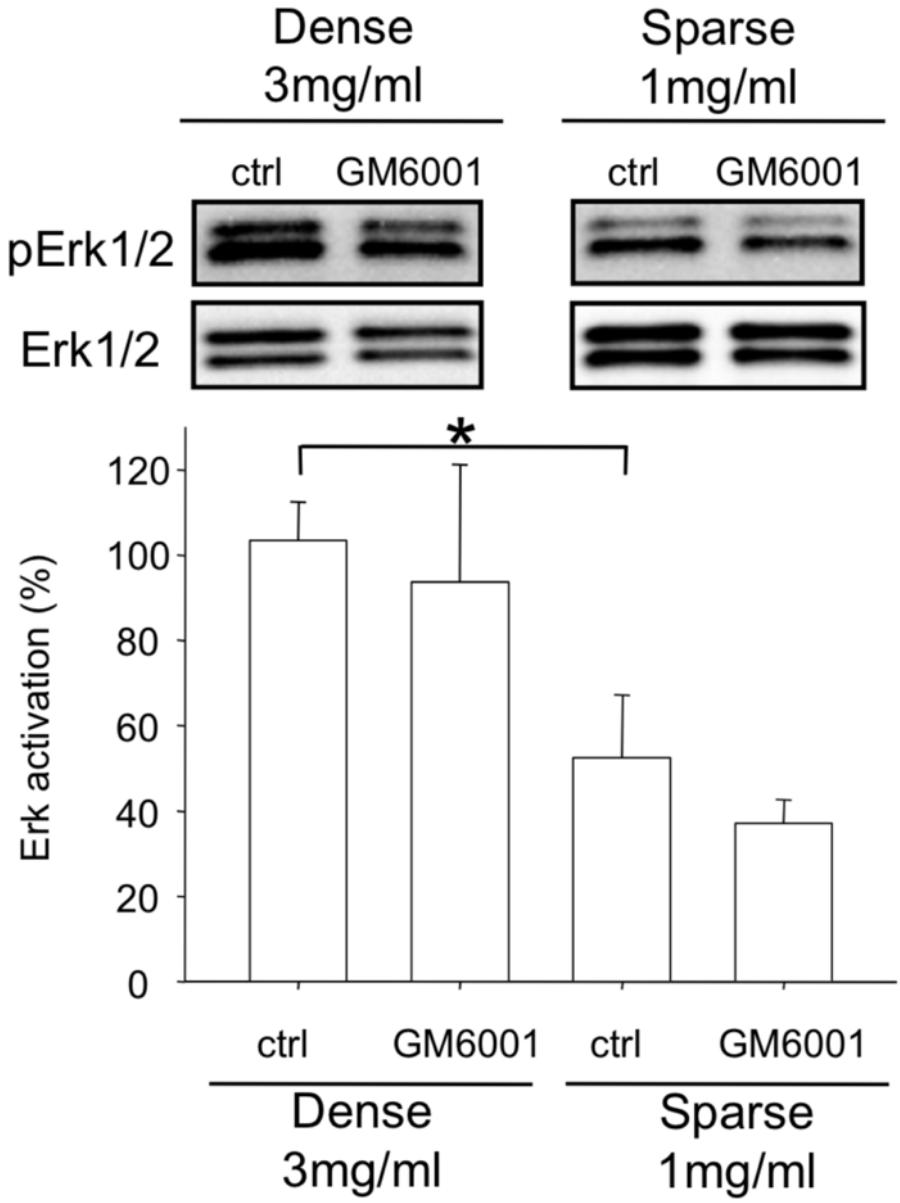

Figure 4.10 ERK1/2 activation of EpH4 cells in dense (3mg/ml) and sparse (1mg/ml) collagen-1 gels cultured for 24 hours with or without GM6001 (40μM). Ratio of activation is quantified and normalized to 100% with (*) indicated p<0.05.

To determine the importance of MMP14 in the regulation of MAP kinase signaling in mammary epithelial cells, we examined Erk activity in EpH4 cells in dense and sparse collagen. Activation of Erk, as defined by its relative phosphorylation, was more dramatic during branching in dense than in sparse collagen, depicted in Figure 4.10.

However, Erk activation was not significantly altered in cultures where GM6001 was present, indicating that MMP proteolytic activity was not specifically required for Erk activation during branching morphogenesis, shown in Figure 4.10. Based on our observations that MMP14 proteolytic activity could be dispensed with but the MMP14 protein could not during branching morphogenesis in sparse collagen-1, we pondered whether MMP14 protein was specifically required. To test this hypothesis, we altered MMP14 expression in EpH4 cells by decreasing MMP14 expression with shRNA. We found that down-modulation of MMP14 with shRNA decreased Erk activity significantly in both dense and sparse collagen-1 gels, whereas exogenous expression of MMP14 increased Erk activity in collagen-1 gels, depicted in Figure 4.11.

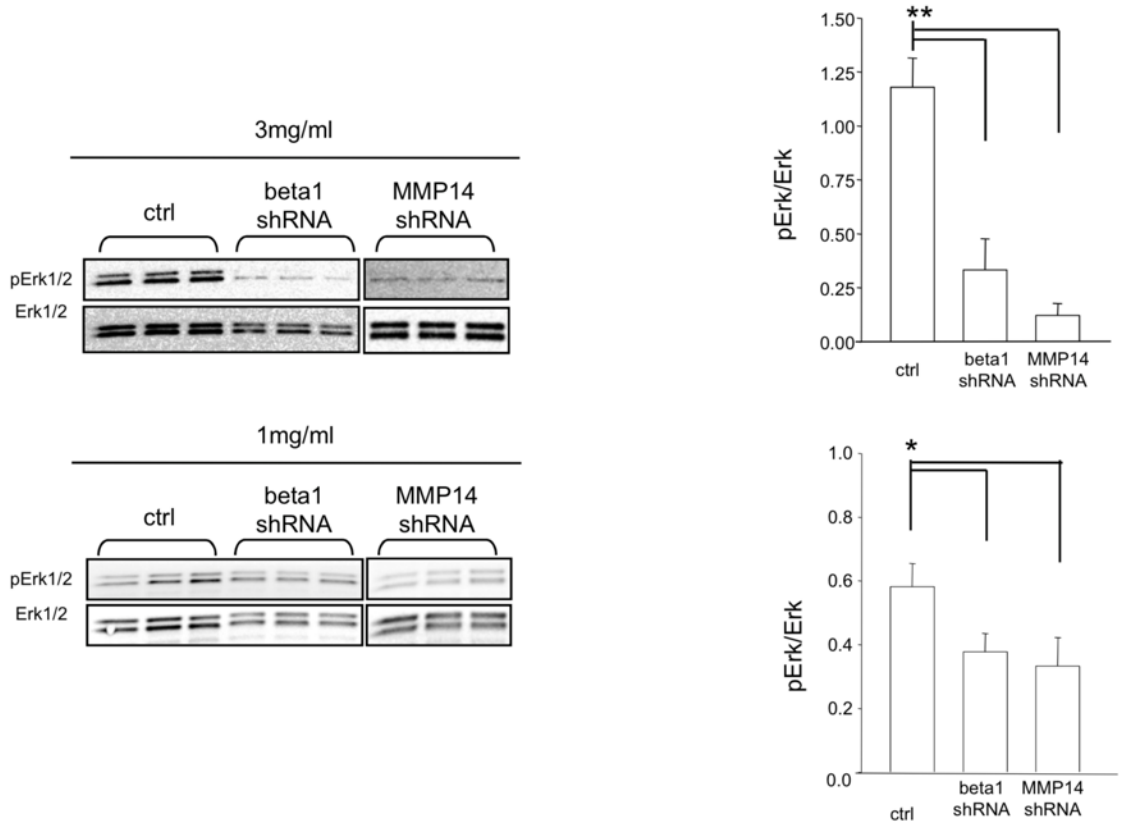

Figure 4.11 EpH4 cells knocked-down either with shRNA against β1-integrin or MMP14 cultured in both dense (3mg/ml) and sparse (1mg/ml) collagen-1 gels decrease ERK1/2 activation, (**) indicating p< 0.01, (*) indicating p<0.05.

Because Erk activity was unchanged with GM6001, this indicated that activation might be dependent on non-catalytic domains of MMP14. This was confirmed when MMP14 dCAT was over-expressed in EpH4 cells. Over expression of either MMP14 wild type or MMP14 dCAT led to higher activation of Erk than observed in control EpH4 cells expressing only the endogenous MMP14, depicted in Figure 4.12.

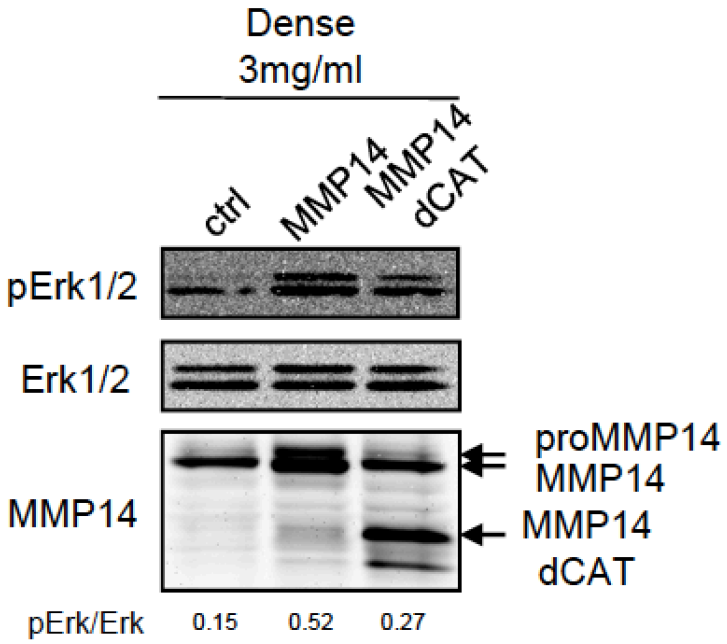

Figure 4.12 EpH4 cells over-expressing either MMP14 or MMP14-dCAT illustrates an increased activation of ERK1/2.

Src activity is also enhanced when MMP14 or MMP14 dCAT are over-expressed in mammary epithelial cells, depicted in Figure 4.13. Down modulation of β1-integrin showed a similar decrease in Erk activity in collagen-1gels as seen in Figure 4.11. These results show that MMP14 and β1-integrin are involved in the control of Erk activation in the context of collagen-1.

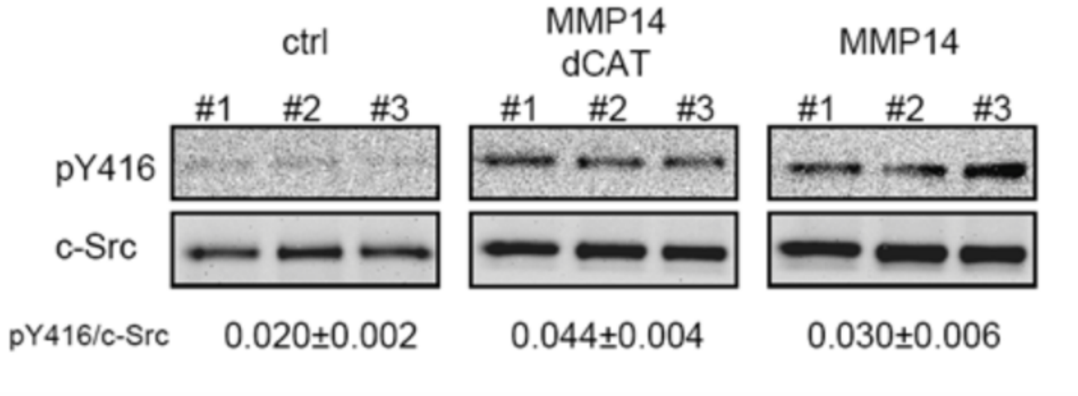

Figure 4.13 EpH4 cells over-expressing either MMP14 or MMP14-dCAT illustrates an increased activation of Src.

4.3.5 – Collagen rigidity and concentration regulates MMP14 expression along with downstream signaling

Once the mammary ducts reach the end of the fat pad the epithelial structures stop invading. This observation suggests that during normal mammary gland development there is a regulator that controls ductal invasion into the stroma and prevents the epithelial structure to invade outside the fat pad. Our results indicate that MMP activity was required for branching only in dense collagen but that the presence of the MMP14 protein was required in both dense and sparse collagen, suggesting that mammary epithelial cells use different aspects of MMP14 under different microenvironmental conditions. To test whether collagen rigidity could modulate levels of MMP14 expression, we altered the stiffness of collagen-1 gels independently of ligand concentration by comparing gels attached to the tissue culture plate with those where the gels were detached and floating (Michalopoulos et al. 1976; Emerman & Pitelka 1977). Atomic force microscopy (AFM) showed that the higher collagen concentration collagen-1 gels are stiffer than lower concentration gels. As such our dense (3mg/ml) gel was significantly stiffer than our sparse (1mg/ml) gel. Subsequently, by floating collagen-1 gels we were able to generate a softer substrate without disrupting the concentration of collagen-1, depicted in Figure 4.14.

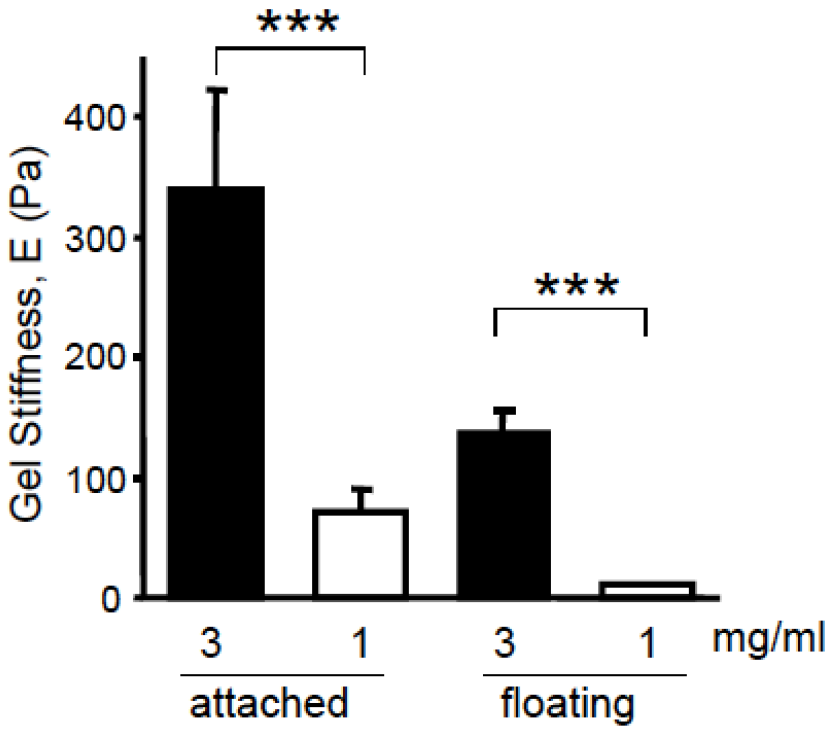

Figure 4.14 Mechanical stiffness of dense (3mg/ml) or sparse (1mg/ml) collagen-1 gels in either a floating or attached model measured by atomic force microscopy (AFM).

After confirming our model to modulate collagen-1 stiffness, we embedded EpH4 cells in the various collagen-1 modalities and isolated RNA to analyze MMP14 expression. Quantitative RT-PCR analysis revealed that MMP14 expression was higher in dense (3mg/ml) compared to sparse (1mg/ml), and attached compared to floated, depicted in Figure 4.15. This concluded that both collagen-1 concentration and stiffness are modifiers of MMP14, and that higher concentration or stiffness will lead to higher MMP14 expression, referring back to Figure 4.10 and 4.11. Interestingly, inhibition of MEK or Src activity in EpH4 cells in dense collagen decrease MMP14 expression

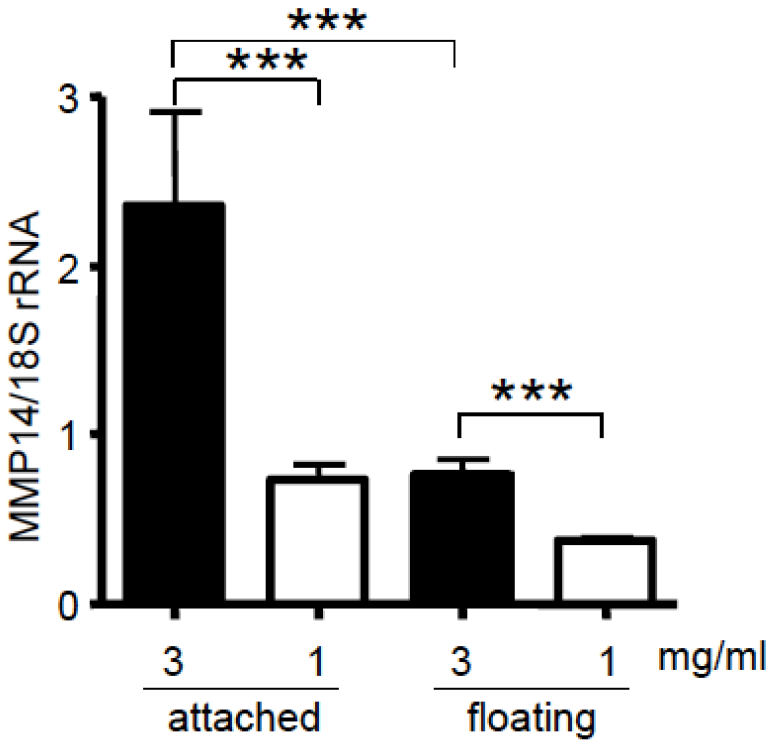

Figure 4.15 MMP14 expression in EpH4 cell cultured in dense (3mg/ml) or sparse (1mg/ml) collagen-1 gels in either a floating or attached model measured quantitative RT-PCR, normalized to 18S, (***) is p<0.001.

A recent study demonstrated that activation of ERK signaling by varying collagen density is potential mechanosensor (Provenzano et al. 2009). Here we demonstrate that MMP14 controls ERK activity and collagen density and stiffness can control MMP14 expression and thus in turn may control ERK activity. Interestingly, inhibition of MEK or Src activity in EpH4 cells in dense collagen decreases MMP14 expression indicating that Src/MEK signaling in turn controls MMP14 mRNA expression, depicted in Figure 4.16. Furthermore, shRNA for β1-integrin also controls MMP14 mRNA expression indicated that engagement of collagen receptor with collagen was required for increased MMP14 expression in dense collagen, depicted in Figure 4.17. These data illustrate the tight reciprocal interactions that are necessary for both increased expression of MMP14, β1-integrin, and activation of ERK activity in a collagen microenvironment.

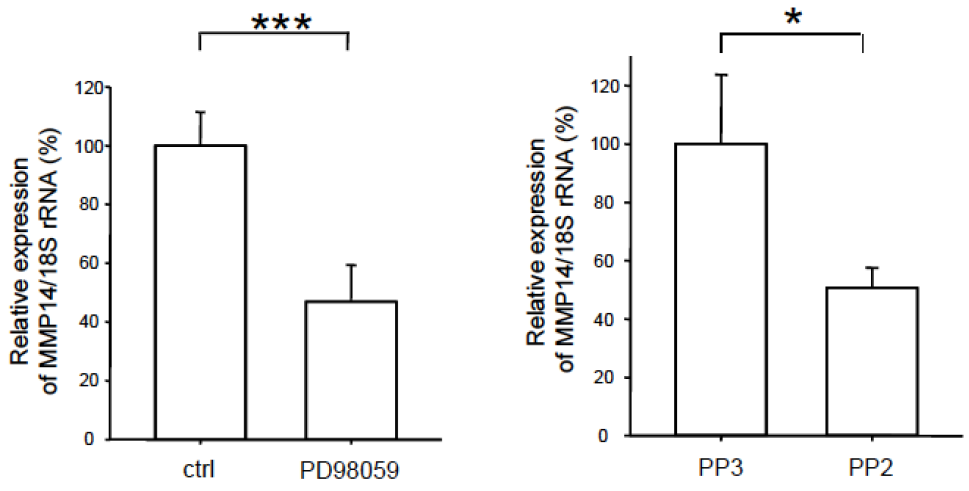

Figure 4.16 MMP14 expression is control by both the MEK and Src pathways illustrated through the quantitative expression of MMP14 normalized to 18S from EpH4 cells cultured in collagen-1 gels treated either with a MEK inhibitor (PD98059), Src inhibitor (PP2) or their associated controls, (*) is p<0.05 and (***) is p<0.001.

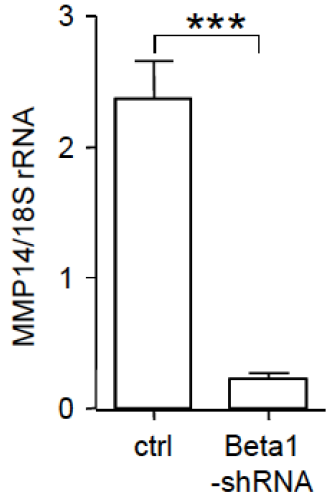

Figure 4.17 MMP14 expression is controlled by β1-integrin illustrated through the quantitative expression of MMP14 normalized to 18S from EpH4 cells cultured in collagen-1 gels, (***) is p<0.001.

4.3.6 – MMP14 controls integrin dependent cell activities in a collagen-1 microenvironment

If the dynamic reciprocal association between MMP14 and β1-integrin is a key regulator for MMP14 function in branching morphogenesis in collagen-1 gels, MMP14 might also be able to control the events that depend on β1-integrin function such as cell spreading and traction. We first determined whether cell-spreading activity is modified in MMP14 or β1-integrin shRNA-treated EpH4 cells. However, neither MMP14 nor β1-integrin shRNA-treated EpH4 cells were able to spread on collagen-1 gels as compared to control shRNA-treated cells, depicted in Figure 4.18. To test whether MMP14 could rescue the cell spreading phenotype in collagen-1 gels, MMP14 was over-expressed in MMP14 or β1-integrin shRNA-treated cells. Over-expression of MMP14 rescued cell spreading in MMP14 shRNA-treated cells, but did not in β1-integrin shRNA-treated cells, depicted in Figure 4.18. This result suggests that MMP14 is able to regulate cell spreading but there is still a requirement for β1-integrin dependent cell adhesion. Interestingly, this MMP14 and β1-integrin dependent cell adhesion was MMP proteolytic activity independent, but was dependent on Src and MEK activity, depicted in Figure 4.19.

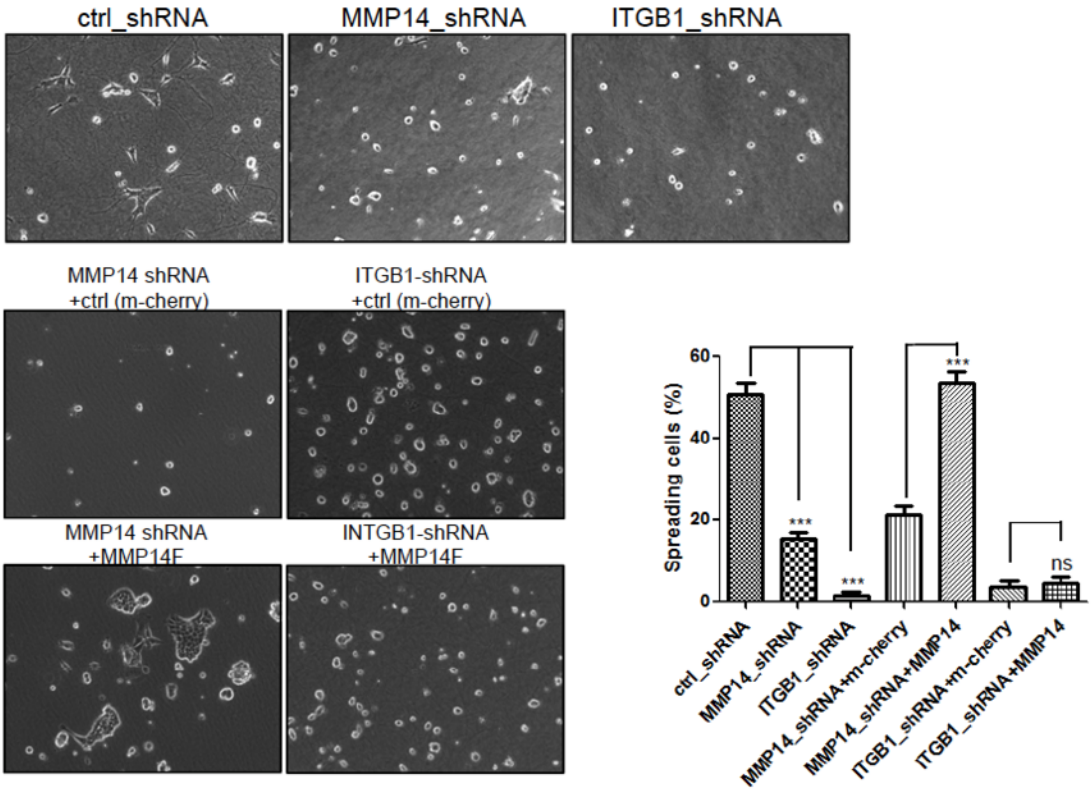

Figure 4.18 MMP14 and β1-integrin are necessary for EpH4 cell spreading on 1mg/ml sparse collagen-1 gels. EpH4 cells are either treated with a control shRNA, MMP14 shRNA, or β1-integrin shRNA. (***) is p<0.001.

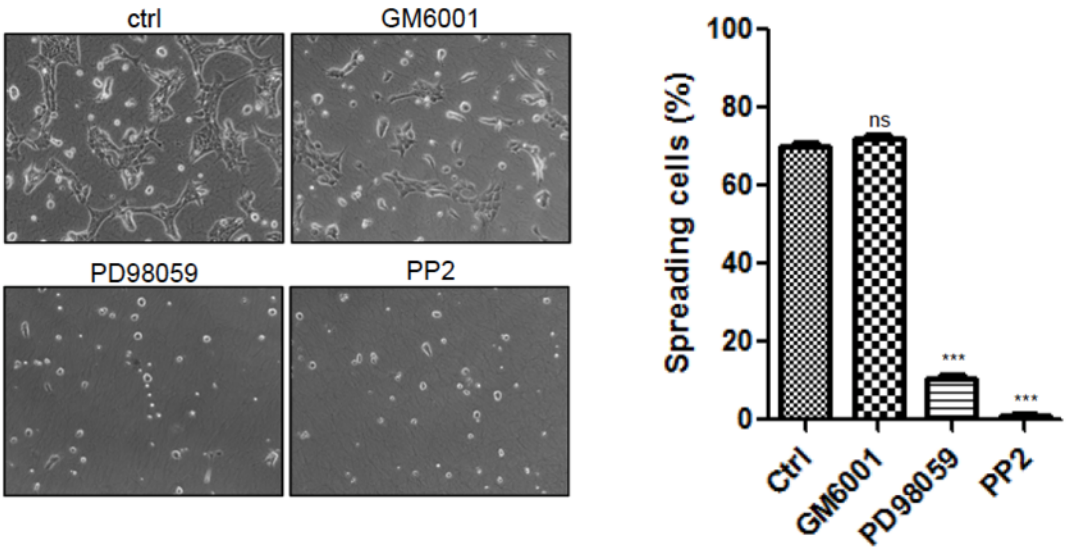

Figure 4.19 MMP14 proteolytic activity is not necessary but MEK and Src activity are necessary for cell spreading on 1mg/ml sparse collagen-1 gels. EpH4 cells treated either with a MMP proteolytic inhibitor (GM6001), MEK inhibitor (PD98059), Src inhibitor (PP2) or a control. (***) is p<0.001.

Next we tested collagen-1 gel traction ability in MMP14 or β1-integrin shRNA-treated EpH4 cells. Similar to the cell spreading assay, MMP14 or β1-integrin shRNA-treated cells reduced the gel traction function, depicted in Figure 4.20. Over-expression of MMP14 rescued the gel traction ability in MMP14 shRNA-treated cells, but not β1-integrin shRNA-treated cells, depicted in Figure 4.20. These results suggested that MMP14 controls collagen-1 gel traction, but β1-integrin function is still required. Again, this collagen-1 gel traction ability was not due to MMP proteolytic activity, but was dependent on Src and MEK activity, depicted in Figure 4.21.

To test the requirement of β1-integrin in MMP14 dependent branching, MMP14 was over-expressed in β1-integrin shRNA-treated cells. Over-expression of MMP14 in β1-integrin shRNA-treated cells did not rescue the branching in 1mg/ml sparse collagen-1 gels, depicted in Figure 4.22. This result is suggesting that MMP14 alone is not sufficient to rescue branching without β1-integrin function. Thus, these data suggests that MMP14 affects β1-integrin dependent cell behaviors in a collagen-1 microenvironment, and that β1-integrin plays a key role in branching morphogenesis.

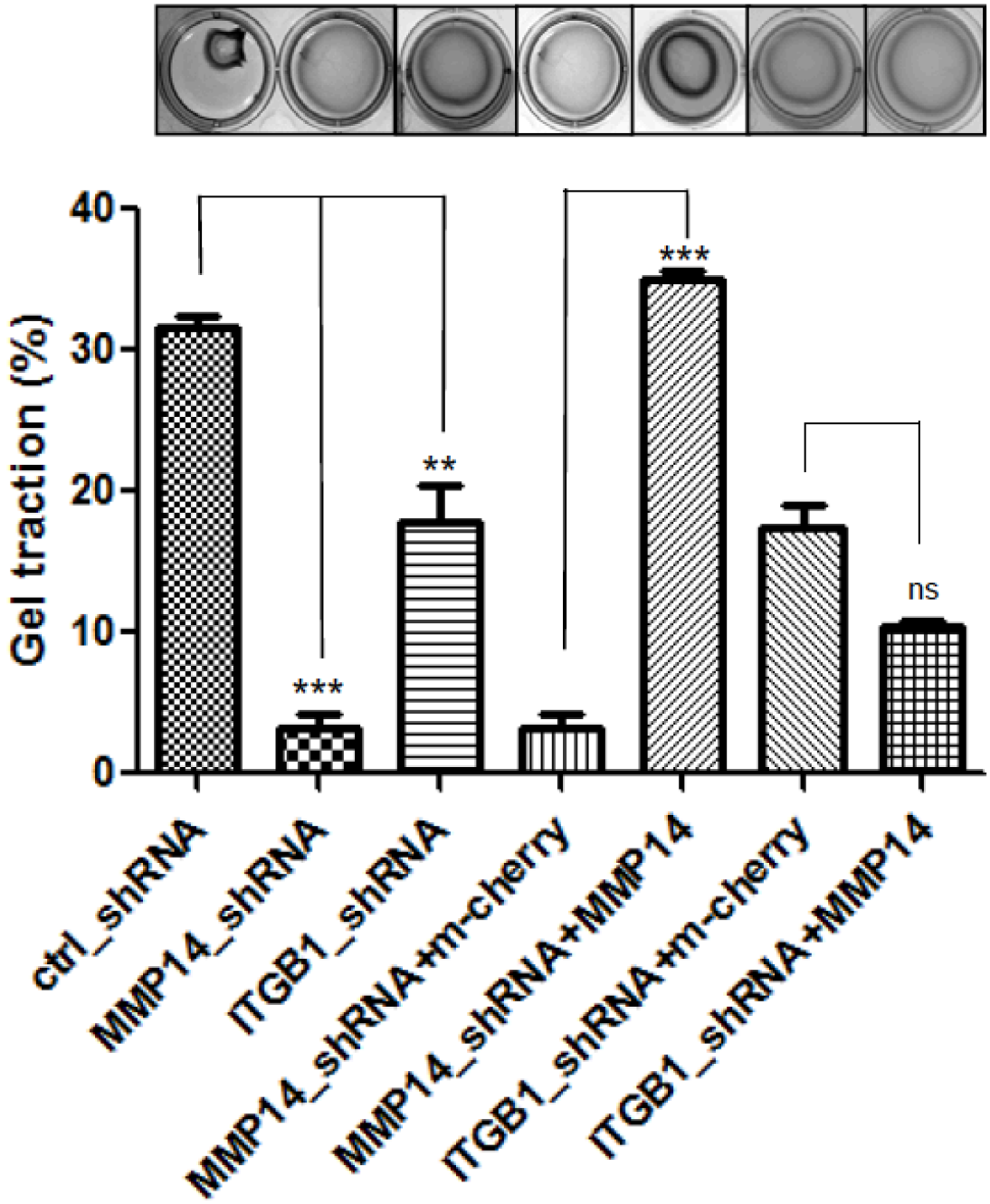

Figure 4.20 MMP14 and β1-integrin are necessary for EpH4 cell traction in sparse collagen-1 gels. EpH4 cells treated either shRNA against MMP14, β1-integrin, or a control cultured on-top sparse collagen-1 gels. Rescue experiments are conducted by over-expressing wild-type MMP14 to the shRNA treated cells. (**) is p<0.01, (***) is p<0.001.

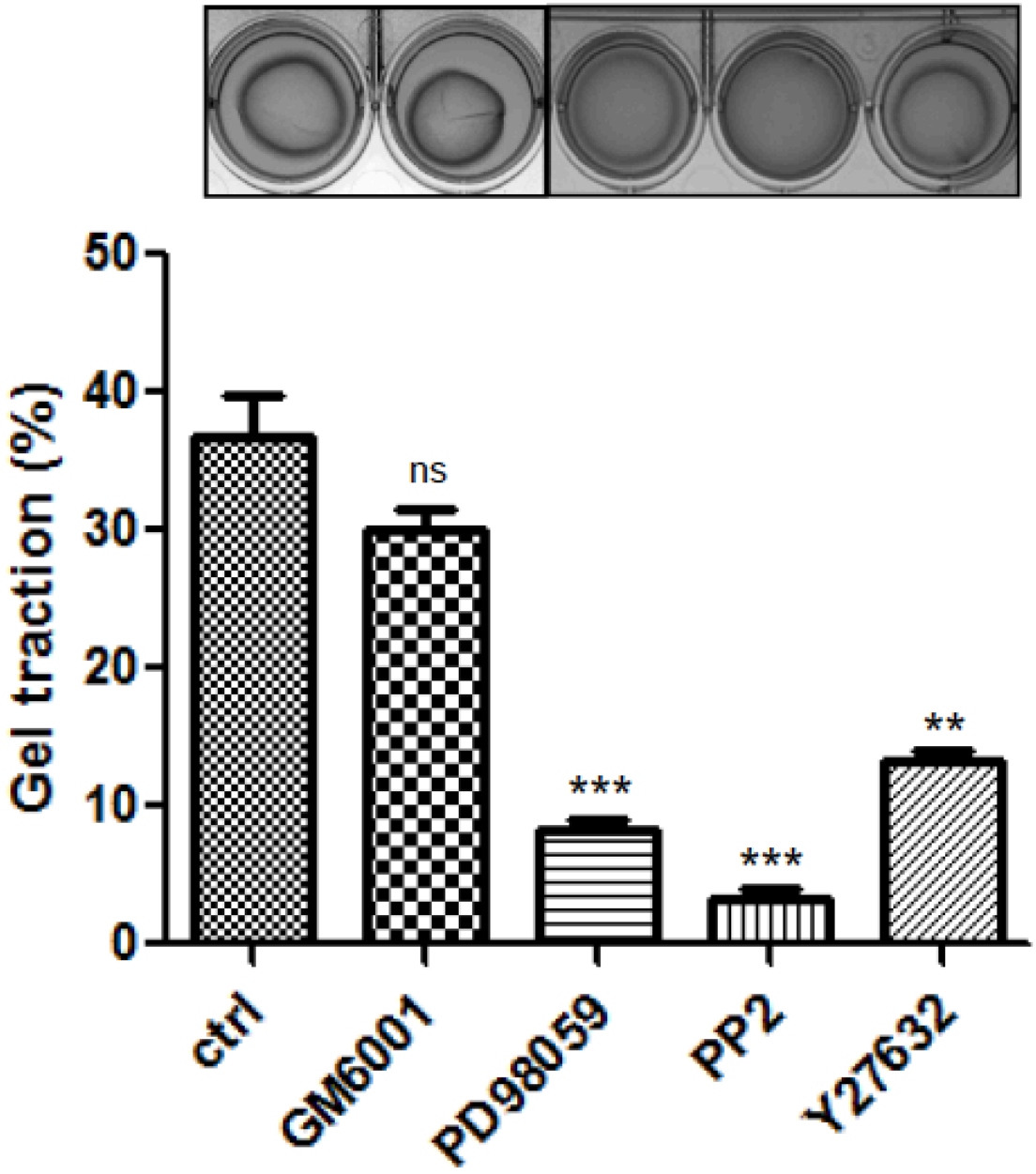

Figure 4.21 MMP proteolytic activity is not necessary, but MEK, Src and Rock activity are necessary for traction in sparse collagen-1 gels in EpH4 cells. EpH4 cells are treated either with MMP proteolytic inhibitor (GM6001), MEK inhibitor (PD98059), Src inhibitor (PP2), Rock inhibitor (Y27632) or a vehicle control. (**) is p<0.01, (***) is p<0.001.

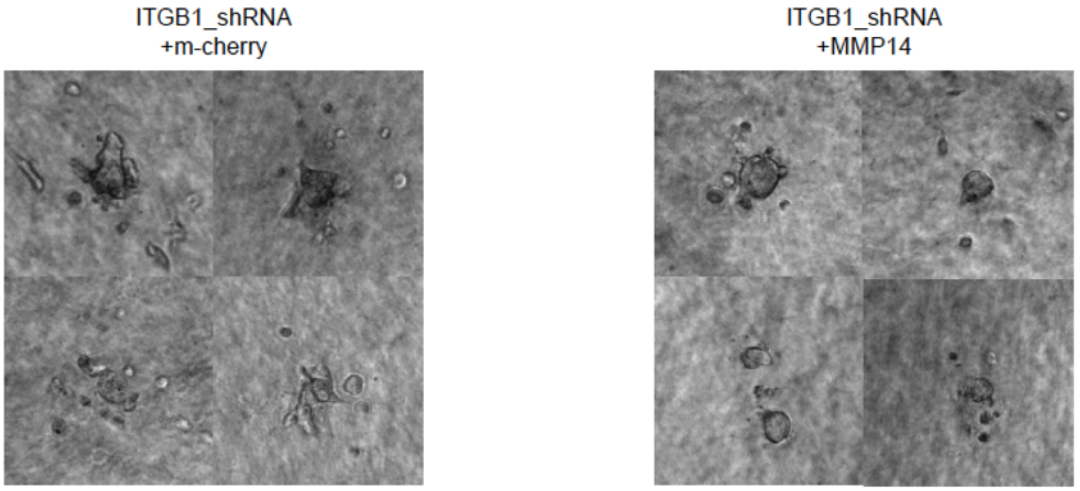

Figure 4.22 β1-integrin is necessary for MMP14 dependent branching in EpH4 cells in sparse collagen. Over-expressing vectors containing either a mCherry control or MMP14 were infected into β1-integrin shRNA treated EpH4 cells demonstrated that MMP14 was unable to rescue the branching in β1-integrin knockdown EpH4 cells.

4.3.7 – Conditional MAPK activation rescues phenotypes of cell spreading, collagen gel traction and branching in MMP14 or β1-integrin shRNA-treated cells

Our data suggests that MAP kinase signaling downstream of MMP14 and β1-integrin is critical for branching morphogenesis. Additionally, over-expression of constitutively active RAS in EpH4 cells enhances cell proliferation in collagen gels and tumorigenesis in the mammary gland and MEK activation in EpH4 cells enhances cell invasiveness, EMT and tumorigenesis in mouse mammary cleared fat pads (E et al. 2002; Pinkas & Leder 2002). Thus, we speculated that MAP kinase activation could rescue the phenotypes of cell spreading, collagen-1 gel traction and branching in MMP14 or β1-integrin shRNA-treated cells. To test this hypothesis, RafER which contains the c-Raf kinase domain and estrogen binding domain to control c-Raf activity, was over expressed in MMP14 or β1-integrin shRNA-treated cells (Samuels et al. 1993). Addition of β-estradiol to these cells increases MAP kinase activity and did indeed rescue the phenotypes of cell spreading and collagen-1 gel traction, depicted in Figure 4.23, 4.24, and 4.25. Moreover, branching morphogenesis was rescued in these cells by activation of RafER, depicted in Figure 4.26. However, many EpH4 cells clusters showed scattering in the collagen gel, we hypothesize that the scattering behavior is dependent on the intensity and timing of the MAP kinase activation by β-estradiol because ERK activation is well regulated in parental EpH4 cells. These results suggest that MAP kinase activity is important to control MMP14/β1-integrin dependent cell behavior in a collagen-1 microenvironment and that a reciprocal interaction exists between MMP14 protein, β1-integrin and MAP kinase signaling pathway to regulate branching morphogenesis.

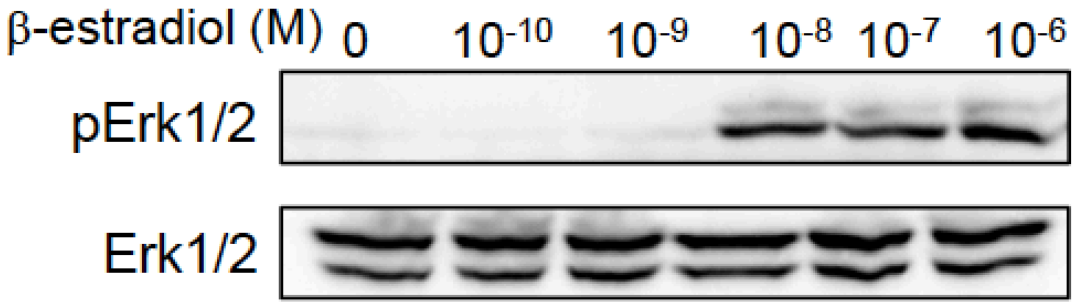

Figure 4.23 Western blotting of phospho-ERK1/2 indicating dose dependent activation of ERK signaling upon addition of β-estradiol to EpH4 over-expressing RafER cells.

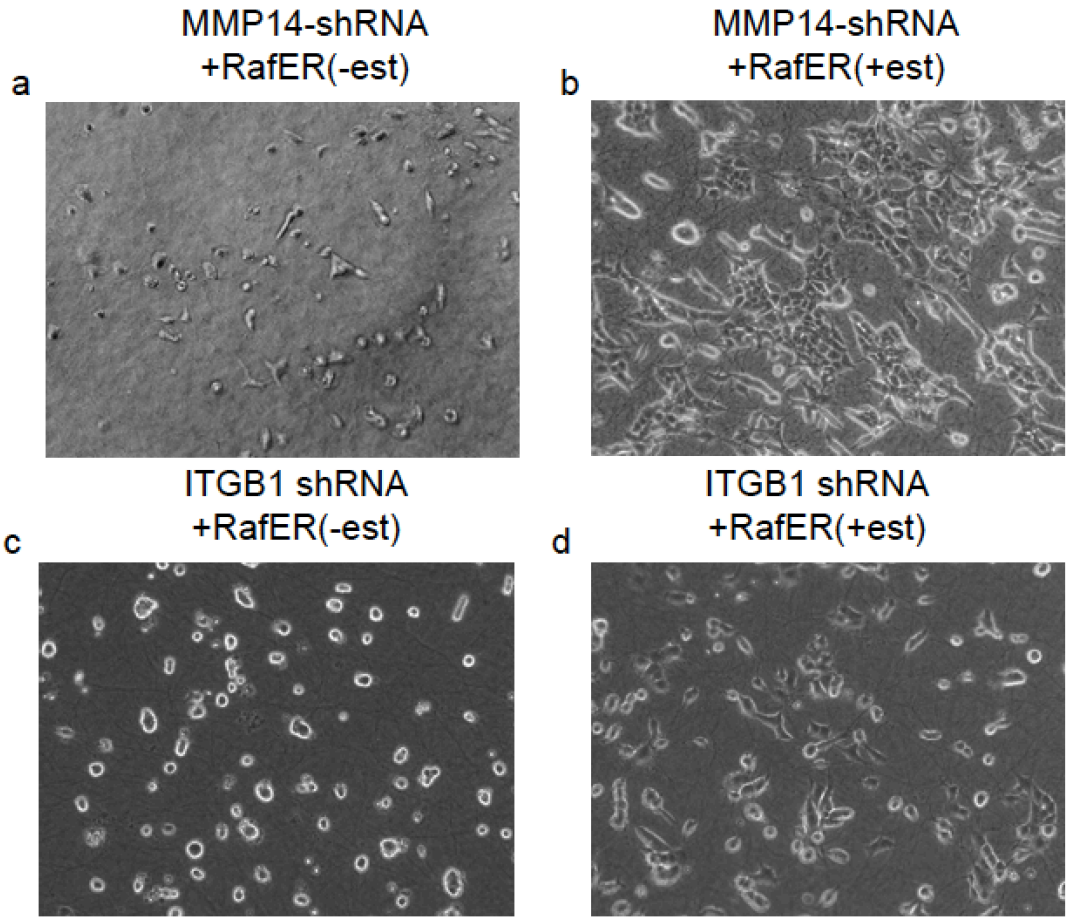

Figure 4.24 Activation of MAP kinase signaling restores cell spreading on sparse collagen-1 gels from loss of either MMP14 or β1-integrin. shRNA MMP14 or β1-integrin treated EpH4 cells were infected with RafER and cultured on sparse collagen-1 gels and treated with β-estradiol to activate MAP kinase signaling.

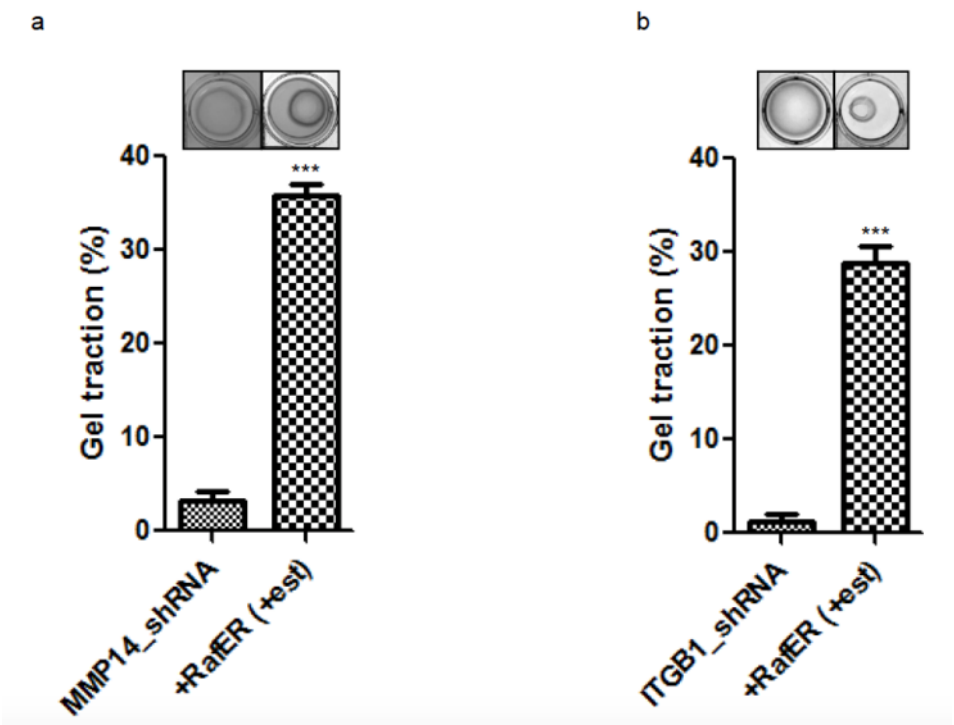

Figure 4.25 Activation of MAP kinase signaling restores cell traction on sparse collagen-1 gels from loss of either MMP14 or β1-integrin. shRNA MMP14 or β1-integrin treated EpH4 cells were infected with RafER and cultured on sparse collagen-1 gels and treated with β-estradiol to activate MAP kinase signaling. (***) is p<0.001.

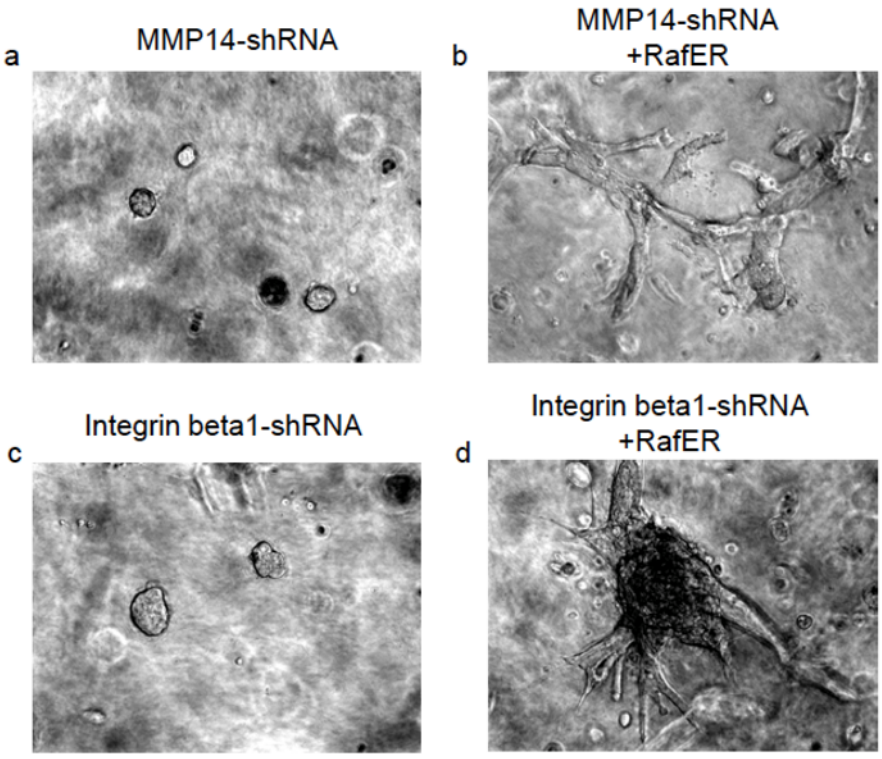

Figure 4.26 Activation of MAP kinase signaling restores mammary epithelial branching in 3D collagen gels from loss of either MMP14 or β1-integrin. shRNA MMP14 or β1-integrin treated EpH4 cells were infected with RafER and cultured on 3mg/ml dense collagen-1 gels and treated with β-estradiol to activate MAP kinase signaling.

4.4 – Discussion

Signaling from the microenvironment drives MMP-mediated ECM remodeling accompanies the dynamic cellular rearrangements that occur during branching morphogenesis in the mammary gland. Integrin mediated signaling and MMP-dependent ECM remodeling are two essential components of this signaling cascade that is required for branching morphogenesis. Here we have investigated and identified two separate requirements for MMP14 and β1-integrin during branching and invasion. We focused on the role of MMP14 due to the observation that into the stroma of the mammary gland: MMP proteolytic activity and MMP14 expression, and downstream signaling of MMP14 are required for branching in 3mg/ml dense collagen-1, but MMP14 proteolytic activity is not required in 1mg/ml sparse collagen-1gels. MMP14 expression is high at the end buds in the mammary gland and at the tips of branching tubules in culture, suggesting that the proteolytic activity of MMP14 is most likely necessary to cleave dense collagen in order to generate a path for the end buds to penetrate into the stroma and for tubules in invade the collagen-1 gel in culture. Using 3D collagen-1 culture models we have identified specific requirements for MMP14 during branching. MMP14 proteolytic activity is necessary when the mammary epithelial cells are in contact with dense collagen but

proteolytic activity is dispensable when the cells are in contact with sparse collagen. However, even though proteolytic activity is not required for branching in sparse collagen there remains a requirement for MMP14 protein expression. Thus, we show that MMP14 expression is necessary for branching, irrespective of its proteolytic activity and the local density of collagen-1, suggesting an essential role for non-proteolytic activity of MMP14.

MMP14 is known to interact with other cell surface molecules, such as integrin-αV and CD44 (Baciu et al. 2003; Mori et al. 2002). It has been suggested that MMP14 modulates the cross-talk between integrin-αVβ3 and integrin-α2β1 on MCF-7 cells, and affects the cell adhesion ability on collagen-1 (Baciu et al. 2003). Furthermore, MMP14 dependent cell migration in COS-1 cells on fibronectin is controlled by cross-talk between the hemopexin domain and catalytic domain of MMP14 (Cao et al. 2004). MMP14 is also known to form oligomers with itself via the hemopexin domain (Itoh et al. 2001). However, how MMP14 oligomers may be involved in the processes characterized here remains to be determined. We recently demonstrated that the interaction between the non-catalytic domain of MMP14 and CD44 is involved in branching morphogenesis by activating downstream signaling pathways of CD44 (Mori et al. 2009). In the current study, we have shown clearly that there is a functional interplay between MMP14 and β1-integirn. Together these observations point to the importance of MMP14 in branching morphogenesis and that its function is not restricted to degradation of ECM molecules during matrix remodeling but that MMP14 also plays an important role signaling via its non-catalytic domains.

Reciprocal signaling from the ECM to the cell is essential for the fine-tuning of cellular behavior during morphogenesis. The collagen concentration surrounding fibroblasts correlates with MMP14 expression. Here we demonstrated an MMP14-dependent event in mammary epithelial morphogenesis in collagen-1 gels is not only dependent on the proteolytic activity of MMP14, but also dependent on the associated function of β1-integrin and its downstream kinase signaling in 3D collagen-1 gels. MMP14 is known to form oligomers with itself via the hemopexin domain and collagen density controls downstream signals of integrins (Itoh et al. 2001; Provenzano et al. 2009). However, it still remains to be solved how these MMP14 oligomerizations are involved in the process. Furthermore, we show that MMP14 decreases when dense 3mg/ml collagen-1 gels are floated, indicating that its expression correlates also with mechanical tension, which is reduced by floatation of the gels. The reciprocal loop that coordinates MMP14 expression with collagen stiffness could demonstrate a strategic mechanism to drive epithelial invasion during mammary branching morphogenesis where collagen concentration is high and where the proteolytic component of MMP14 is needed. However, we (in this study) and others have shown that proteolytic activity is dispensable during invasion of single breast cancer cell or MMP14 over-expressing osteosarcoma cells in pepsin treated collagen-1 gels (Wolf et al. 2003). On the other hand, proteolytic activity and MMP14 expression were indispensable for collective cell movement of these cells under the same conditions (Wolf et al. 2007). Thus, MMP14 proteolytic and non-proteolytic activities might not only be utilized in normal branching, depending on the local density of the stroma but also in different modes of cell migration used by malignant cells. Our finding that migration

through sparse collagen-1 gels still requires MMP14 but not its proteolytic activity suggests an additional adhesive and/or signaling role for the non-proteolytic MMP14 domains during mammary ductal invasion. Whereas these two modes of MMP14 action are reminiscent of the tumor migration modes described by others, there are also distinct differences between the branching strategies uncovered here and tumor cell migration in collagen-1 gels, namely that neither ECM ligation nor that MMP14 can be dispensed with and β1-integrin is always required (Friedl 2004).

Recently, the differences between pepsin treated and acid extracted collagen on cell migration were tested. Cell invasion was stopped with an MMP inhibitor or MMP14 siRNA in acid extracted collagen, but not in pepsin treated or crosslinking inhibited collagen (Sabeh, Li, et al. 2009). In our study, we used acid extracted collagen-1 to mimic conditions similar to those found in vivo, and demonstrated MMP14 proteolytic activity dependent or independent modes of branching in normal mammary epithelial cells by using different collagen densities. The findings discovered by others and by our lab suggest that the differences in migration are context-dependent.

Intracellular signaling cascades are essential for mammary epithelial cells to respond to the microenvironmental stimuli directing morphogenesis. For example, c-Src (-/-) mice have defects in mammary ductal elongation and ERK signaling is essential for morphogenesis in several models, including branching of the prostate using organoid models, tubular formation in collagen-1 gels using MDCK cells, alveologenesis of mammary epithelial organoids in Matrigel and morphogenesis of blood vessel networks on Matrigel (Kim et al. 2005; Kuslak & Marker 2007; O'Brien et al. 2004; Fata et al. 2007; Maru et al. 1998). MMP14 was shown to modulate cellular invasion by activating ERK in COS-7 and fibrosarcoma cells (Gingras et al. 2001; Takino et al. 2004). An intriguing implication of our findings is that while MMP14 affects the function of integrin and regulates MAP kinase activation in normal mammary epithelial cells in a 3D collagen-1 microenvironment, the opposite is also true, meaning inhibition of MEK and Src activities not only inhibit branching, but also affect MMP14 and β1-integrin expression. Here we connect these signaling pathways to each other and to the density of stromal collagen, shedding light upon the fact that collagen density and rigidity tune MMP14 expression through integrin and Src/MEK pathways, and MMP14 expression affects β1-integrin expression and its downstream signals. We illustrate in our study the increased expression of MMP14 at the tips of invading mammary end buds and others have shown this to be true in other tissues such as the ureteric buds and angiogenic sprouts (Kanwar et al. 1999; Meyer et al. 2004; Hotary et al. 2002). This localization of MMP14 expression in the invading front might serve to localize Src/ERK signaling to the invasive areas explaining the requirement for MMP14 even without the need for its proteolytic activity. Although MMP14 (-/-) mice were reported to undergo essential normal embryonic development, the analysis of postnatal mammary gland has indicated a severe defect in ductal invasion providing additional support for our findings that MMP14 is required for invasion through collagenous stroma of the postnatal mammary gland (Holmbeck et al. 1999; Kanwar et al. 1999).

The data presented here points out to a unique interplay between MMP14 and β1-integrin. Illustrating not only is β1-integrin necessary for invasion through collagen-1 gels, it is also required for collagen-1 induced expression of MMP14. These results demonstrate the requirement for β1-integrin in epithelial cells. A comparable result is found to be true in fibronectin and collagen mediated induction of MMP expression in fibroblasts (Huhtala et al. 1995; Langholz et al. 1995). Our data supports the large number of function blocking studies in vivo and in culture suggesting an absolute requirement for β1-integrin in mammary epithelial branching (Berdichevsky et al. 1994; Alford et al. 1998; Klinowska et al. 1999). The surprising finding is that a conditional knockout of β1-integrin in the mouse mammary gland of FVB mice using the MMTV promoter allows essentially normal branching morphogenesis which may be explained by the fact that MMTV expression is mosaic in this model or that the compensation of β1-integrin by other integrins, such as β3-integrin is able to rescue the branching phenotype (White et al. 2004; Wagner et al. 2001). Alternatively and equally plausible are strain-specific differences in morphologies of mammary epithelial trees (Naylor & Ormandy 2002). These findings further support our results that branching mechanisms depend on the biochemical and mechanical properties of the stromal microenvironment which vary greatly depending on strain, age, hormonal status, and most probably also the immunological status of the host mammary gland. The reciprocal interactions described in this work underscores the utility of the physiological environment of three-dimensional assays, as such the use of such assays in conjunction with engineered mice help elucidate and unravel the complex signaling interactions of the many regulators of ECM receptors and the interplay between signaling ligands during mammary gland development.

Cell migration and invasion in collagen has been discussed in this manuscript and numerous studies performed by other investigators. Studies have been completed investigating how the proteolytic activity of MMPs was dispensable during invasion of a single breast cancer cell or how MMP14 over-expressing osteosarcoma cells in pepsin treated collagen-1 gels was indispensable for collective cell movement (Wolf et al. 2003; Wolf et al. 2007). These papers suggest to us that MMP14 proteolytic and non-proteolytic activities might not only be utilized in normal branching depending on local density of the stroma, but also in different modes of cell migration used by malignant cells. Our findings indicate that migration through 1mg/ml sparse collagen-1 still requires MMP14 but not its proteolytic activity, suggesting an additional adhesive and/or signaling role for the non-proteolytic activity of MMP14 during mammary gland ductal invasion. Additionally, we demonstrated that MMP14 affects β1-integrin function in normal mammary epithelial cells in 1mg/ml sparse collagen-1 providing more evidence to suggest such as role. Whereas these two modes of MMP14 action are reminiscent of the tumor migration modes described by other researchers, there are also distinct differences between the branching strategies uncovered here and tumor cell migration in collagen-1 gels, namely that neither ECM ligation nor MMP14 can be dispensed with and at least β1-integrin is always required (Friedl 2004). Recently, the differences between pepsin-treated and acid-extracted collagen were highlighted in previous publications demonstrating that cancer cell invasion was stopped with MMP inhibitors or MMP14 siRNA in acid-extracted

collagen but not in pepsin treated or crosslink inhibited collagen (Wolf et al. 2003; Sabeh, Li, et al. 2009). Here we used acid-extracted collagen-1 to mimic similar conditions in vivo demonstrating MMP14 associated mammary epithelial branching can be dependent or independent of proteolytic activity depending on the collagen-1 density. Our findings and the findings of others suggest that the differences are context-dependent.

Concentration of collagen was found to correlate with MMP14 expression in fibroblasts and that collagen density controls downstream signals of integrins (Tomasek et al. 1997; Provenzano et al. 2009). Here we connect the two findings by showing that the epithelial expression of MMP14 also correlates with the density and rigidity of the collagen matrix. Importantly, we demonstrate that the collagen content during mammary gland development in vivo parallels the expression of MMP14 supporting the physiological significance of our finding (Keely et al. 1995). Finally, we show that MMP14 decreases when 3mg/ml dense collagen-1 gels are floated indicating that its expression correlates also with mechanical tension, which is reduced by the flotation of the gels. The reciprocal loop that coordinates MMP14 expression with collagen stiffness is a strategic mechanism to drive epithelial invasion during mammary branching morphogenesis at dense collagen areas.

Mammographic breast density is a significant risk factor for cancer and tissue stiffness can promote tumor development and invasion (Boyd et al. 1998; Boyd 2007; Paszek et al. 2005). MMP14 is up-regulated often within invasive breast cancers(Ueno et al. 1997). Our data illustrates that up regulation of MMP14 by dense collagen may provide a mechanistic explanation for why increased tissue stiffness and increased stromal ECM correlate with breast cancer risk (Gilles et al. 1997; Paszek et al. 2005). We showed previously that MMP3 can induce chromosomal aberrations and mammary tumors in transgenic mice, and that up regulation of MMP3 in epithelial cells leads to genomic instability and epithelial mesenchymal transformation via induction of reactive oxygen species (Sternlicht et al. 2000; Radisky et al. 2005). These findings, combined with our results of occurrence of vimentin expression on the tip of branching structures, the requirement of MMP14 in mammary invasion into the fat pad, and the influence of collagen density on the level of MMP14 expression could provide another possible link between MMPs and mammary cancer (Nelson et al. 2006). We suggest that a similar coordination between tissue density and MMP14 could result in a positive feedback loop that propels breast tumor progression.

Chapter 5 – The relationship between TGF-β1 and modulation of branching morphogenesis in relationship to tumor susceptibility

5.1 – Introduction

Exposure to natural and occupational hazards including chemicals and low-dose ionizing radiation (LDIR) is an unavoidable consequence of living in the natural world and modern industrial societies. Assessment of possible oncogenic risks resulting from LDIR exposures forms an essential part of radiation protection strategies. Whereas extrapolation analysis from high-dose exposures provides a frame of reference for the entire population, it cannot provide adequate risk assessment at the level of the individual. Epidemiological and genetic studies show that there is a strong genetic component that contributes to the differences between individuals in their response to LDIR exposure and cancer susceptibility. An understanding of individual susceptibility across life span to cancer risk will facilitate prevention and decrease public health burden.

Efforts underway to detect genetic variants in human populations are likely to be fraught with difficulties. The genetic heterogeneity and variable etiology of carcinogenesis in humans will necessitate collection of large numbers of DNA samples from cancer patients and control populations, with no guarantee that the methods presently available allow detection of the most important loci. Furthermore, the inability to quantify the levels of environmental carcinogens to which different individuals are exposed adds a further dimension of uncertainty to the resolution of these questions. Because of these problems, genetic studies to identify susceptibility loci have largely been limited to investigating candidate genes involved in response to DNA damage. Parallel studies in mice offer many advantages for the study of the genetic basis of complex traits (Balmain 2002; Mao & Balmain 2003), including LDIR-induced cancers. Our ability to control genetic background and to carry out crosses between mouse strains differing in their propensity to develop these diseases offers unprecedented opportunities to identify and investigate the primary genetic loci that control susceptibility. In addition, studies with mice allow precise exposures, standardized husbandry to control other environmental components of risk, and comprehensive analysis of phenotypes.

Cancer is an aberrant growth with malignant (i.e. invasion and metastasis) properties in the context of the physiology of a complex organism, thus many other factors in addition to mutation play important roles in its induction and progression. The pathophysiology of cancer, as that of any other organ, depends not only on the intrinsic properties of the parenchymal component (tumor cells), but also on other organismic compartments including stroma (Donjacour & Cunha 1991), extracellular matrix integrity (M. J. Bissell & Radisky 2001), and the immune, endocrine and vascular systems (Pérez-Losada et al. 2011) All play key roles in the development of cancer. Consequently, radiation cancer susceptibility, specially the role of low dose, is not only going to be determined by canonical factors traditionally measured only in tumor cells, such as proliferation, apoptosis, and DNA repair, but also will be influenced by the tumor cell's microenvironment (Kessenbrock et al. 2010; M. J. Bissell & Hines 2011). Indeed radiation-induced changes in the stromal microenvironment can contribute to

neoplastic progression in vivo (Barcellos-Hoff & Ravani 2000; Nguyen, Oketch-Rabah, et al. 2011). Furthermore, the two main compartments of the mammary gland (epithelia and stroma) are not independent, but rather continuously crosstalk and interact with each other, so that the intrinsic factors are capable of recruiting the extrinsic factors, and the nature of the signaling of the extrinsic factors determines the intrinsic cellular activity i.e. there is a continuous dynamic reciprocity between cells and their surroundings. Importantly, radiation can affect both compartments (Maxwell et al. 2008).

BALB/c is sensitive to mammary carcinogenesis and radiation exposure enhances this susceptibility (A. C. Blackburn et al. 2003; Okayasu et al. 2000; Yu et al. 2001). Prior work has attributed the BALB/c sensitivity to a polymorphism in DNA-PKcs and hence, to misrepair of radiation damage. However, our recent study showed that radiation operates via the microenvironment to accelerate Trp53 null mammary carcinogenesis (Nguyen, Oketch-Rabah, et al. 2011), suggesting that there are multiple genetic determinants. In contrast, SPRET/EiJ is wild, recently inbred mice that are resistant to radiation or other environmental factors induced carcinogenesis, and its backcross with mus musculus strains has proved useful for identifying SNPs associated with disease states (Balmain 2002; Mao & Balmain 2003; Pérez-Losada et al. 2011). Here we used the radiation chimera model (Nguyen, Martinez-Ruiz, et al. 2011) and the backcross strategy to identify the genetic variations that affect microenvironments and subsequently regulate Trp53 null carcinogenesis in irradiated mice.

5.2 – Methods

Mice

SPRET/EiJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine). The female interspecific F1 hybrid mice between BALB/c, a susceptible strain to radiation-induced mammary tumor development and SPRET/EiJ, a resistant strain, were crossed with male BALB/c to generate F1 backcross (F1Bx) mice (BABL/c x SPRET/EiJ) x BALB/c. These mice were used as hosts for the radiation chimera model (12). In 10- to 11-week-old F1Bx host mice, the inguinal fat pad was divested of endogenous mammary epithelium and then transplanted with a Trp53 null mammary fragment. Half of the mice received 10 cGy of whole-body radiation 3 days prior to transplantation of the Trp53 null fragments. Mice were monitored and palpated for mammary tumor development. Tumor growth was determined with digital calipers, and tumor volume was estimated every week by the known formula: *Tumor volume = length x width^2 x 0.5* (Euhus *et al.,* 1986; Tomayko and Reynolds, 1989). Animal treatment and care was carried out in accordance with the animal protocols and approved by the Animal Welfare and Research Committee at Lawrence Berkeley National Laboratory.

Blood collection
Blood was collected at 6 hr and 15 weeks after radiation or SHAM treatment in EDTA coated collection tubes and was processed by centrifugation at 1500g at 48C in a refrigerated microfuge for 10 min. Plasma was transferred and aliquoted to a fresh RNase/DNase free 1.5 microfuge tube. Plasma samples were stored at -80^{o}C for metabolite and cytokine profiles.

Plasma cytokine assay
Blood was collected by retro-orbital bleed from mice at 6 hr and 15 weeks after LDIR or sham- treatment in EDTA coated collection tubes and was processed by centrifugation at 1500g at 48C in a refrigerated microfuge for 10 min. Plasma was transferred and aliquoted to a fresh RNase/DNase free 1.5 microfuge tube. Plasma samples were stored at -80^{o}C for cytokine profiles. Plasma diluted 1:1 was run in triplicate on 32 premixed Milliplex™ Cytokine Kit plates (Millipore) following the standard protocol. Samples are incubated overnight, processed and were run on a Luminex 200™. 32 mouse cytokines and chemokines include: Eotaxin, G-CSF, GM-CSF, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1α, MIP-1β, MIP-2, RANTES, TNFα, VEGF.

Mammary gland tissue collection and wholemounts
At the time of dissection, the stage of estrous was determined by vaginal lavage followed by cytological analysis. For each study, the thoracic and inguinal mammary glands were excised and frozen immediately in freezing medium (90% fetal bovine serum and 10% dimethyl sulfoxide) for organoid cultures, banked in optimum cutting temperature (OCT) compound (Tissue-Tek) for immunohistochemistry, or they were formalin fixed for histological analysis. One inguinal gland was fixed in Carnoy's solution for 30 minutes then stained with carmine alum overnight to analyze ductal/alveolar morphology. Histomorphometry to compare differences in epithelial density was performed using a mammary wholemount. Quantification of epithelial density was done via an image processing and analysis program (ImageJ; National Institutes of Health). To quantify bifurcation points, each branch beginning from the nipple were counted on each mammary gland wholemount. H&E staining were generated by the UCSF Helen Diller Family Comprehensive Cancer Center Mouse Pathology Core.

Determination of secreted TGF-β1
Organoids were grown in lrECM three-dimensional culture and seeded at 200 per well in a 48-well plate in serum-free media. Cells were treated with TGFα for 48-hours and the amounts of total and active TGFβ were determined using a commercial TGFβ1 enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen).

Immunofluorescent staining
Serial formalin fixed paraffin tissue sections (thickness: 5 μm) of mammary glands were generated by the UCSF Helen Diller Family Comprehensive Cancer Center Mouse Pathology Core. Mammary sections were deparaffinized and rehydrated in graded

alcohol to 1X PBS, followed by heat-mediated antigen retrieval in Tris-EDTA Buffer (10mM Tris Base, 1mM EDTA, 0.05% Tween-20, pH 9.0) using a vegetable steamer. Slides were then permeabilized with 0.2% Triton-X and blocked for 1 hour in 10% serum. Slides were incubated overnight with either a polyclonal chicken antibody against active TGFβ1 antibody (R&D Systems AF-101-NA, 10) or a polyclonal goat antibody against LAP TGFβ1 (R&D Systems AB-246-NA, 50). Secondary antibodies used were rabbit anti-chicken 568 or 488 and rabbit anti-goat 488 or 568 (Invitrogen). Tissues were imaged on a Zeiss LSM 710 confocal microscope using a 0.45 NA x10 air objective. Quantification was done as previously described (Burgess et al. 2010).

Western blotting

Mouse organoids were lysed in 2% SDS/PBS. 30 µg of protein of each lysate was then separated on a Tris-glycine 4-20% gel. phospho-p44/42 was probed with a mouse monoclonal antibody (Cell Signaling 9106, 1:250), p44/42 was probed with a rabbit polyclonal antibody (Cell Signaling 9102, 1:1000), phospho-SMAD2 was probed with a rabbit polyclonal antibody (Cell Signaling 3104), and SMAD2 was probed with a rabbit monoclonal antibody (Cell Signaling 5339). The blot was stripped and re-probed with a rabbit polyclonal antibody against α-tubulin, used here as a loading control (Abcam ab18251, 1:1000).

Statistical and linkage analysis

The Kaplan–Meier and Cox regression method was used to compare the tumor latency while the Chi-square test was used to compare mammary tumor frequency between 10cGy or SHAM treated mice. Statistical analysis was performed using SPSS version 12.0 (SPSS, Chicago, IL). Linkage analysis was carried out using R/QTL.

5.3 – Results

5.3.1 – Study design for a systems genetics analysis of stromal microenvironment in mammary tumor susceptibility to LDIR

To examine the effects of LDIR and the stromal microenvironment on mammary tumor development, the endogenous epithelium was surgically removed from F1Bx female mammary glands at 3-weeks of age, and at 11~12 weeks, mice were either irradiated with 10cGy X-rays (LDIR) or sham treated. Three days later, un-irradiated inguinal mammary gland fragments from BALB/c *Trp53* null (p53-/-) mice were transplanted into the LDIR- and sham-treated F1Bx hosts. Mice were monitored for tumor development by palpation for 18 months. Upon detection, tumor growth rate was measured. To measure cytokine levels in plasma, blood was collected from all mice by orbital bleeds at 6 hours and 15 weeks after radiation exposure. Graphically depicted in Figure 5.1 is a cartoon illustration the study design to analyze the genetics networks involved with cancer susceptibility.

Figure 5.1. A study design for systems genetics analysis of mammary tumor susceptibility by radiation exposure

5.3.2 – Mammary tumor development in genetically diverse hosts is reduced by LDIR

In a previous study, LDIR exposure of inbred susceptible BALB/c hosts decreased tumor latency and increased the frequency of tumor incidence for implanted *Trp53* null epithelia (Nguyen, Oketch-Rabah, et al. 2011). Surprisingly, we found that the frequency of *Trp*53 null tumors in LDIR treated F1Bx hosts was *reduced* by 12.3% (p=0.036) compared to sham-irradiated hosts by the time the experiment was terminated as depicted in Figure 5.2. Moreover, irradiation of F1Bx host significantly *delayed* tumorigenesis as depicted in Figure 5.2. However, once the tumors were formed, the rate of tumor growth was increased in irradiated F1Bx host mice relative to sham as depicted in Figure 5.3, as reported previously (Nguyen, Oketch-Rabah, et al. 2011). Over 80% of tumors were estrogen receptor (ER) positive and this frequency was not altered by host irradiation as depicted in Figure 5.4. The majority of tumors were adenocarcinoma (~50%) or squamous cell carcinoma (~40%), the remaining tumors were spindle cell carcinoma, and the distribution of histological types was unaffected by host irradiation as depicted in Figure 5.4. We conclude that introgression of the SPRET/EiJ genome with BALB/c resulted in reduced frequencies and increased latency of mammary tumors in low dose irradiated hosts.

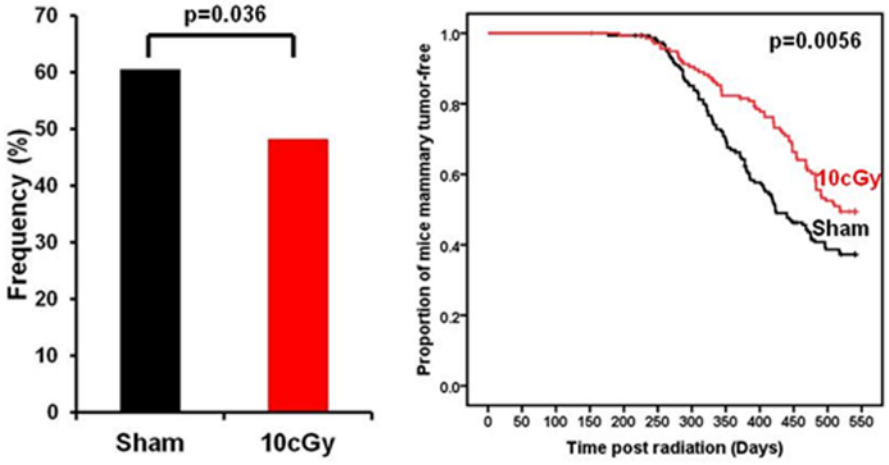

Figure 5.2. The effect of LDIR on tumor phenotypes illustrating LDIR reducing the incidence of mammary tumors and delaying their rate of appearance.

5.3.3 – Identification of genetic loci that control mammary tumor latency

We devised our system analysis of susceptibility to determine how the genetics of the host affects mammary tumor development and progression of *Trp53* null epithelial fragments in F1Bx mice. We interrogated the genetic loci associated with tumor latency and frequency by genome-wide genotyping using Illuminal SNP microarrays. Only two loci, one on chromosome 2 (LOD score=4.26) and the other on chromosome 14 (LOD score=3.43) were associated with tumor latency in sham-treated mice, depicted in Figure 5.5 & 5.7. Mice with homozygous BALB/c alleles at these two loci had significantly shorter latency than those that were heterozygous (one allele from BALB/c and one from SPRET/EiJ) depicted in Figure 5.7, suggesting that some SPRET/EiJ genes delay tumor development in exogenously-grafted BALB/c *Trp53* null epithelial fragments. In contrast, we identified 15 genetic loci that interact with LDIR exposure to control tumor latency, depicted in Table 5.1. The SPRET/EiJ allele in 11 of these 15 loci confers reduced risk to tumor development after host exposure to LDIR depicted in Figure 5.7 and Table 5.1, whereas 4 remaining SPRET/EiJ alleles confer increased susceptibility. These results suggest that host genetic variants strongly influence mammary cancer latency after exposure to LDIR. The observation that many more genetic loci were found in the LDIR cohort compared to the sham cohort suggests a strong host genetic contribution specific to radiation. Given that the host, but not cells producing the tumor, were irradiated, the genetic contribution to mammary tumor susceptibility involves non-cell autonomous mechanisms, which are clearly through host microenvironment.

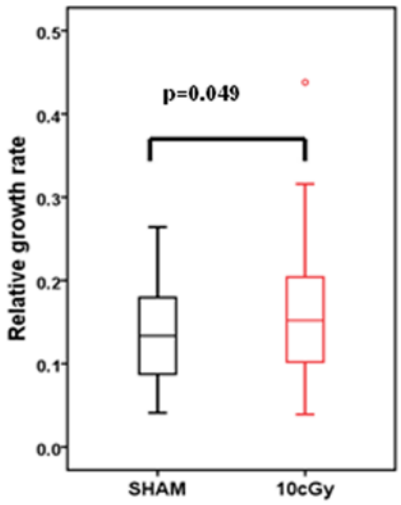

Figure 5.3. The effect of LDIR on tumor phenotypes illustrating LDIR increasing the rate of tumor growth.

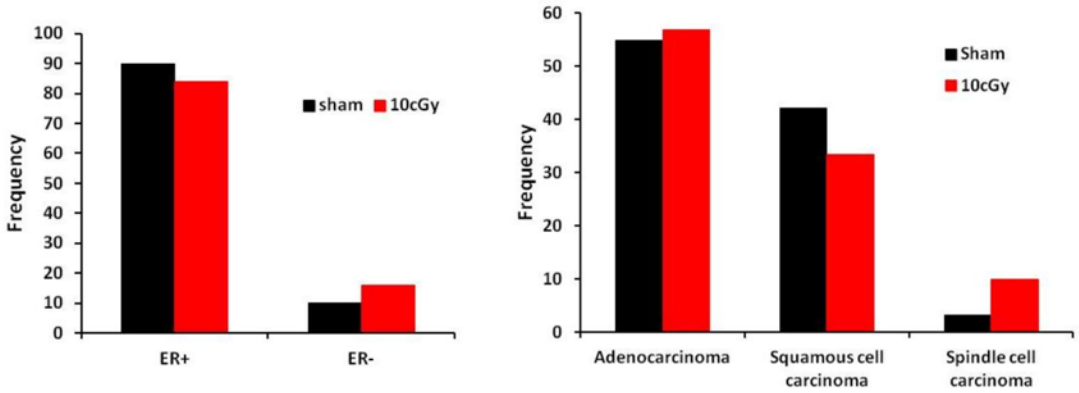

Figure 5.4. The effect of LDIR on tumor phenotypes illustrating LDIR not effecting the distribution of tumor types observed.

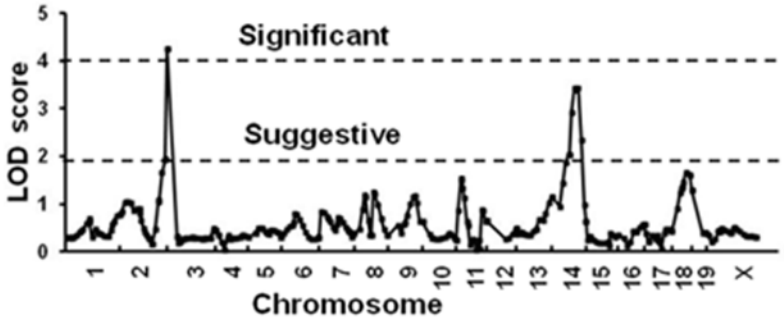

Figure 5.5. Genome-wide LOD scores for tumor latency of Sham-treated mice

To further explore genetic associations with cancer risk after exposure to LDIR, we discovered 696 candidate genes located within 5 Mb of the peak of the identified loci and used Ingenuity Pathway Analysis (IPA) to examine pathways enrichments. Of these, 185 genes were within 4 loci on chromosomes 2, 11, 14, and 16, where homozygous BALB/C alleles are associated with increased cancer latency after exposure to LDIR, and were enriched in four pathways, γ-glutamyl cycle, leukotriene biosynthesis, alanine biosynthesis III and glutathione biosynthesis, depicted in Figure 5.6. In contrast, 511 genes were within 11 regions where the heterozygous SPRET/EiJ allele is associated with increased latency after LDIR treatment, and were enriched for 24 pathways. Importantly these 11 loci were enriched for genes involved in regulating the immune response including signaling pathways of natural killer cells, cytokines, etc. Analysis of the upstream regulators of these candidate genes indicated that the TGFβ (SMAD3) and p53 (CDKN2A) pathways are likely to be involved in mammary tumor susceptibility in response to LDIR, depicted in Figure 5.8.

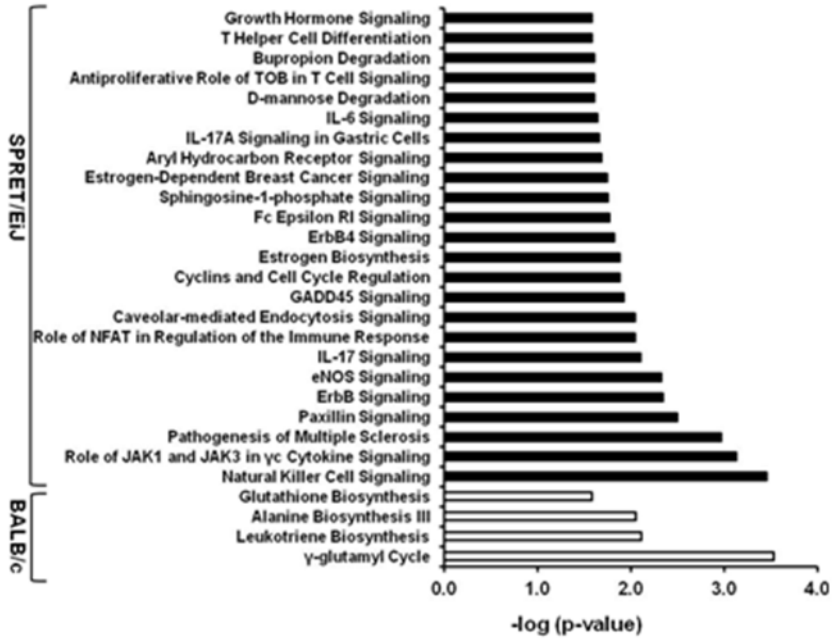

Figure 5.6. Ingenuity Pathway Analysis of potential signaling pathways that were enriched among the candidate genes located within the identified loci.

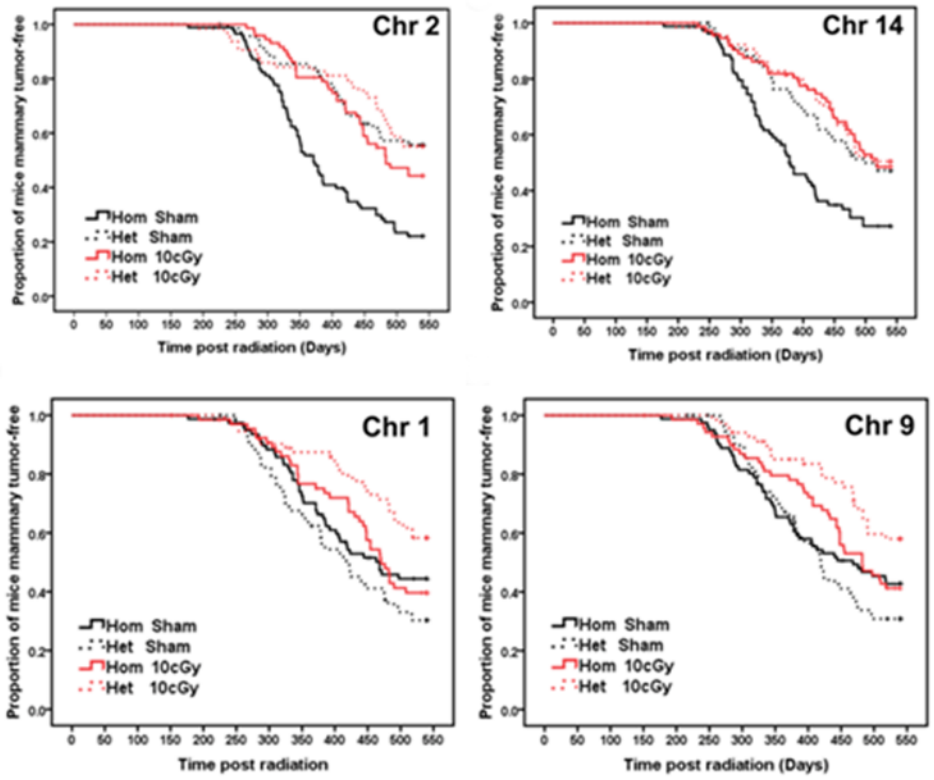

Figure 5.7. Kaplan-Meier curves for tumor latency at the locus of Chromosome 2, 14, 1, and 9.

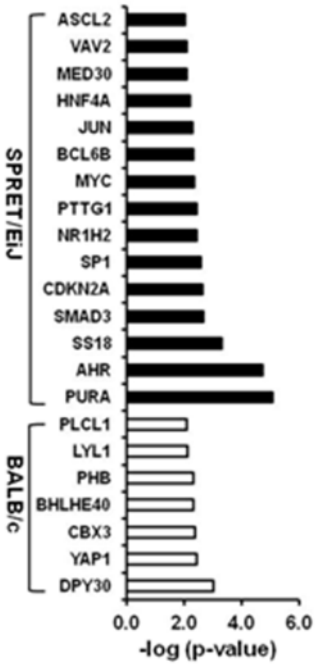

Figure 5.8. Ingenuity Pathway Analysis of transcriptional factors regulating the candidate genes within the identified loci

5.3.4 – Association of plasmas cytokine levels with tumor latency

Given the strong representation of cytokine signaling pathways within the identified stromal genetic loci, we assessed the association with tumor latency by dividing plasma cytokine levels into tertiles (low = bottom third, moderate = middle third, and high = top third). In sham-treated F1Bx mice, plasma levels of eotaxin at 6 hrs after treatment and IL-1A at 15 weeks after treatment were significantly associated with tumor latency. In 10cGy-treated F1Bx mice, plasma levels of two cytokines (G-CSF and IL-13) at the early time point and three cytokines (IP10, LIX, and RANTES) at the later time point were significantly associated with tumor latency. For example, mice with high levels of LIX or RANTES developed tumors significantly later than those with low levels, depicted in Figure 5.9.

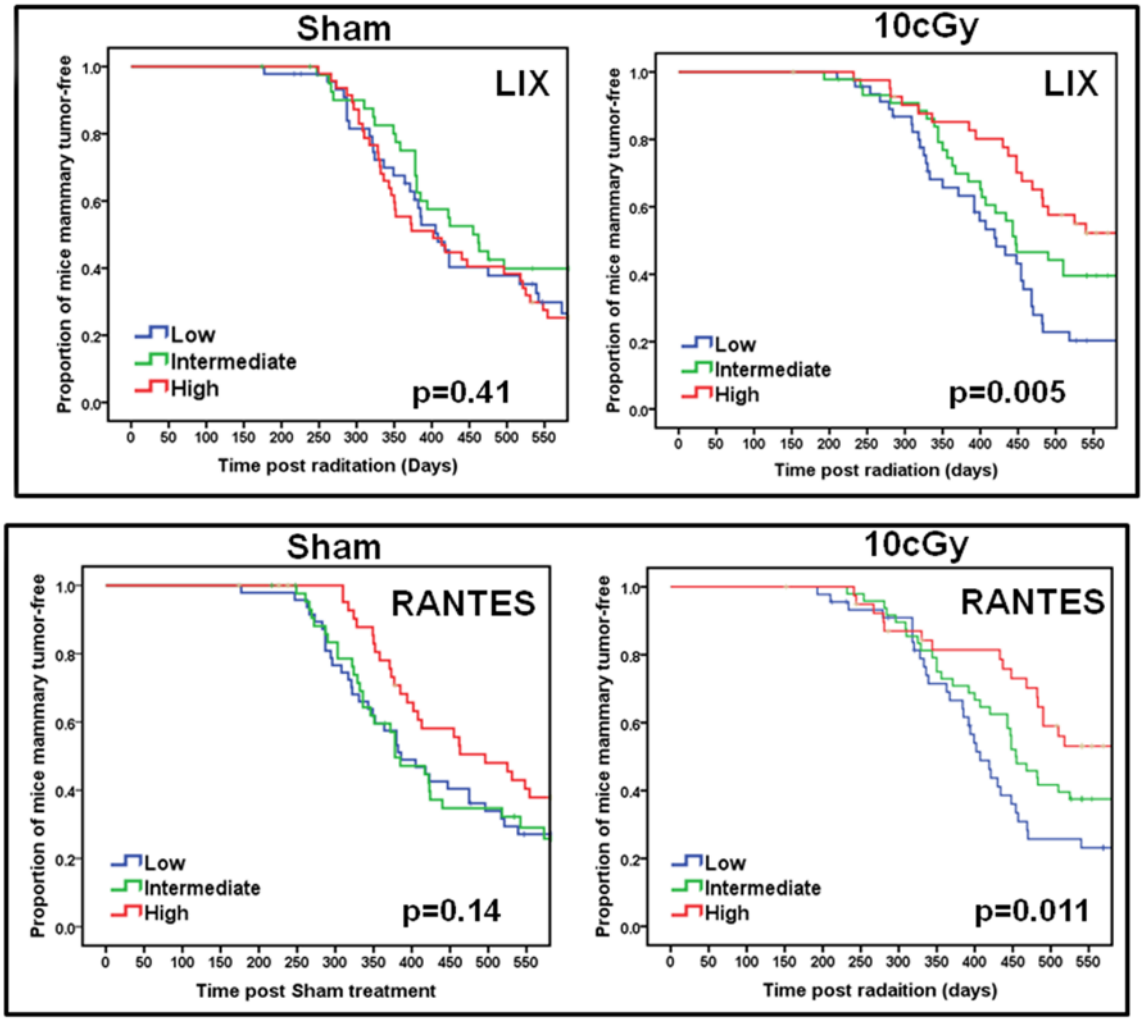

Figure 5.9. Association of plasma cytokine levels of LIX and RANTES with tumor latency

5.3.5 – Mammary gland development differs in BALB/c and SPRET/EiJ mice

To elucidate functional mechanisms of these mammary cancer susceptible loci, we further examined whether BALB/c and SPRET/EiJ parental strains display any differences in normal mammary development. Mammary glands were collected from BALB/c and SPRET/EiJ mice at different ages, then stained with carmine alum to visualize the spatial arrangement of their ductal tree in whole mounts. We found that SPRET/EiJ mammary glands have fewer branches in comparison to BALB/c mammary glands further confirmed by quantitative analysis of the area occupied by epithelial cells and branching at 10 weeks after birth, depicted in Figure 5.10. The morphology of mammary glands from F1 hybrids between BALB/c and SPRET/EiJ mimicked SPRET/EiJ, suggesting that the SPRET/EiJ genome is dominant for this phenotype.

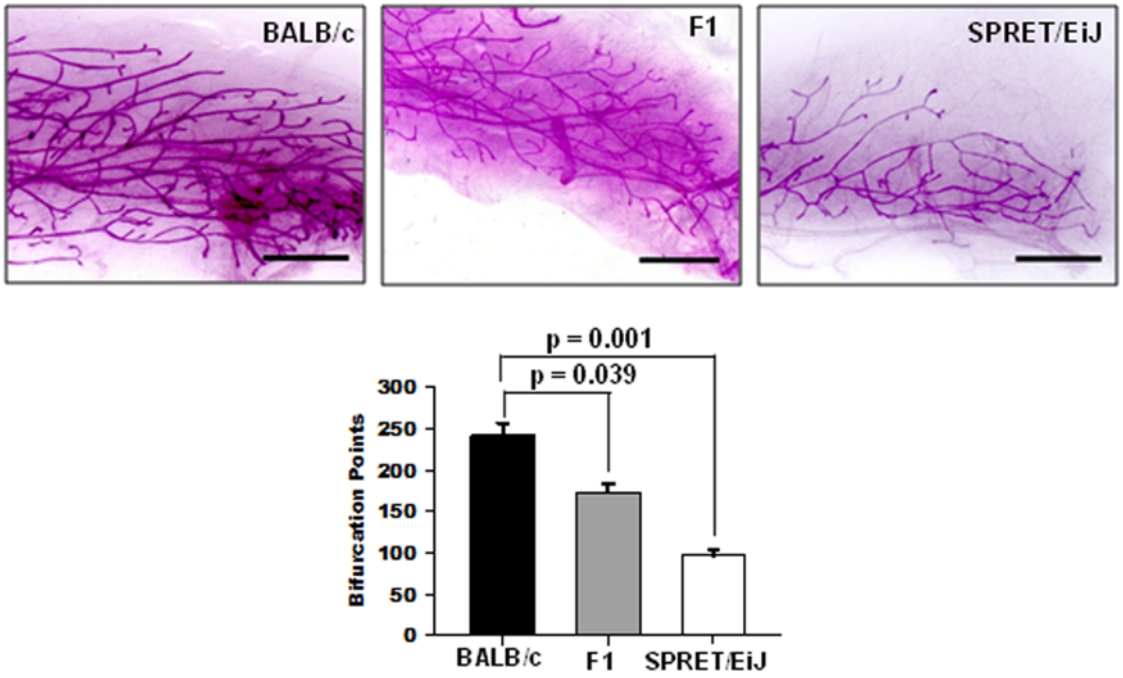

Figure 5.10. Effect of host genetic background on mammary gland architecture, mammary gland wholemounts of BALB/c, BALB/c x SPRET/EiJ (F1), and SPRET/EiJ and quantification of branching.

5.3.6 – TGF-β1 signaling regulates stromal invasion in mammary organoids

Parental strain differences in branching and invasion into the fat pad during mammary development led us to investigate which regulators of these physiological processes differ between BALB/c and SPRET/EiJ. Our SNP mapping and bioinformatic analysis identified the TGFβ pathway, a known regulator of mammary branching and development (Nelson et al. 2006), as a potential mediator of mammary tumor susceptibility. We further examined the involvement of TGFβ1 signaling on breast cancer risk using an ex vivo

organoid culture model to assess stromal invasion (Lo et al. 2012). Ductal fragments from BALB/c, SPRET/EiJ and their F1 hybrid mammary glands were cultured in growth factor-reduced Matrigel and the number of branching organoids were quantified, depicted in Figure 5.11. We observed that SPRET/EiJ and F1 hybrid organoids were unable to form any branched structure, whereas BALB/c organoids branched as reported previously; similar results were obtained using standard 3D culture conditions in 3 mg/ml collagen I. These results suggest that SPRET/EiJ and BALB/c epithelial cells sense and respond to their microenvironments differently.

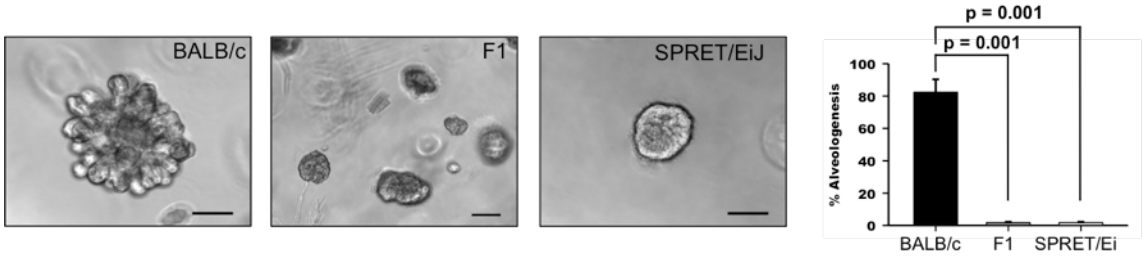

Figure 5.11. Three-dimensional culture in lrECM of BALB/c, BALB/c x SPRET/EiJ (F1), and SPRET/EiJ mammary organoids and quantification of branching.

We also observed that TGFβ1 concentrations measured in culture media were significantly higher when isolated from the SPRET/EiJ organoids compared to BALB/c, suggesting a more direct link between TGFβ1 levels and branching inhibition. Furthermore, assessment of the localization of active and inactive TGF-β1 in developing mammary glands showed a significant increase in active TGF-β1 in the SPRET/EiJ mammary glands compared to BALB/c, depicted in Figure 5.12. Finally, addition of SPRET/EiJ culture media inhibited branching of BALB/c organoids, and the effect was significantly reduced after treatment with a TGFβ1 blocking antibody, depicted in Figure 5.13. These results support the idea that higher TGFβ1 levels in SPRET/EiJ restrict branching, possibly leading to protection against radiation-induced cancers. Further studies are required to determine the precise relationship between ex vivo ductal branching and tumor susceptibility.

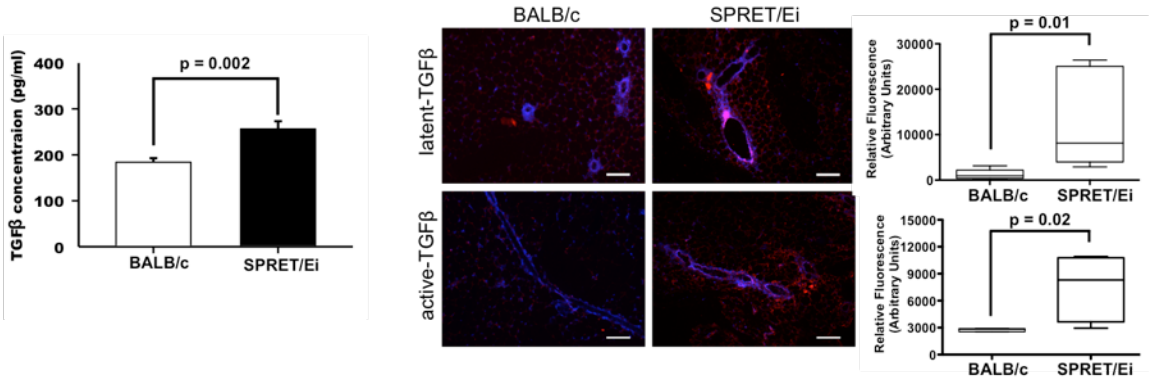

Figure 5.12. Quantification of TGF-β1 levels within BALB/c and SPRET/EiJ through ELISA of culture media and immunohistochemistry of paraffin tissue sections.

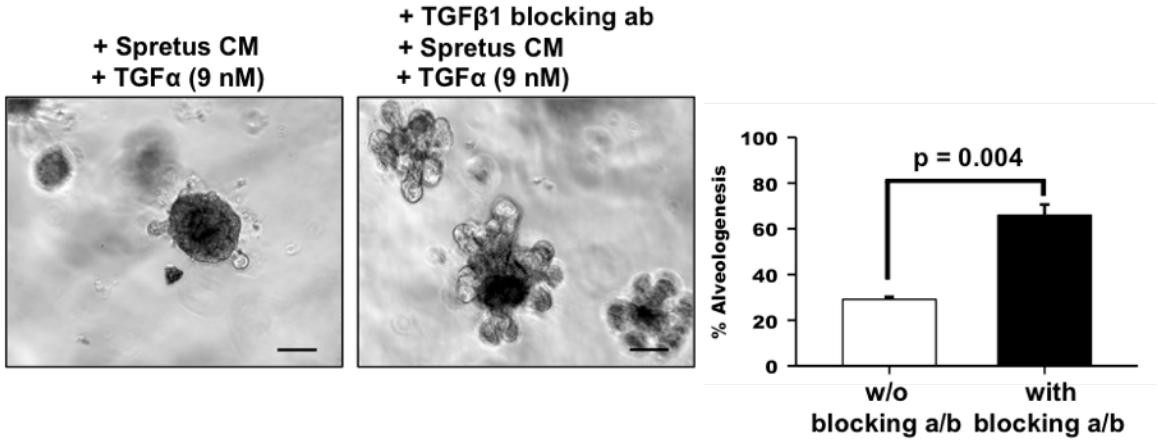

Figure 5.13. Inhibition studies of TGF-β1 levels on BALB/c organoids from SPRET/EiJ culture media.

Table 5.1: Genetic loci intact with LDIR controlling susceptibility to mammary tumor development

Chromosome	Location (Mb)	SNP ID	Homozygous				Heterozygous			
			p-value	HR*	95% CI for HR#		p-value	HR	95% CI for HR	
					Lower	Upper			Lower	Upper
1	174.28	rs13476248	0.97	0.99	0.64	1.54	1.86E-04	0.42	0.27	0.66
2	154.20	rs6376291	2.19E-04	0.47	0.32	0.70	0.88	0.96	0.57	1.61
3	33.93	rs6371982	0.76	0.89	0.44	1.83	2.70E-03	0.59	0.41	0.83
3	148.94	rs3657112	0.60	1.20	0.61	2.38	7.43E-04	0.54	0.38	0.77
4	8.73	rs13477549	0.41	0.83	0.53	1.29	3.09E-03	0.51	0.33	0.80
5	93.65	CEL-5_93652588	0.32	0.81	0.53	1.23	3.92E-03	0.50	0.31	0.80
7	114.58	rs13479513	0.80	0.91	0.44	1.88	2.52E-03	0.58	0.41	0.83
8	56.55	rs3685424	0.99	1.00	0.44	2.28	1.83E-03	0.58	0.41	0.82
9	55.40	rs13480208	0.69	0.92	0.60	1.40	4.07E-04	0.43	0.27	0.68
11	73.72	rs13481099	3.14E-03	0.52	0.33	0.80	0.36	0.81	0.52	1.27
12	96.44	rs3700012	0.36	0.81	0.52	1.27	0.48	0.85	0.55	1.33
14	78.69	rs3708535	6.81E-04	0.48	0.31	0.73	0.57	0.87	0.55	1.40
15	72.19	rs13482641	0.57	0.87	0.55	1.40	1.66E-03	0.56	0.39	0.80
16	50.66	rs4186129	4.34E-03	0.54	0.35	0.83	0.38	0.81	0.50	1.30
17	46.77	rs13483012	0.87	0.94	0.47	1.91	2.83E-03	0.59	0.41	0.83

*HR= Hazard Ratio for 10cGy vs Sham. #CI = Confidence Interval

Table 5.1. Genetic loci intact with LDIR controlling susceptibility in the mammary gland

5.4 – Discussion

In this study, we developed a model system to investigate how genetic variation affects the tumor susceptibility of transplanted *Trp53* null mammary epithelia in response to host irradiation. In this model system, genetically identical and oncogenically primed *Trp53* null mammary cells from inbred BABL/c mice were transplanted into genetically heterogeneous hosts (F1Bx mice) to separate radiation effects on promotion via stroma from direct initiation via DNA damage. We showed that LDIR *reduced* the incidence and *increased* the latency of *Trp53* null mammary tumors in F1Bx hosts. These results differ from previous observations in inbred BALB/c mice, where 10cGy increased the incidence and decreased the latency of *Trp53* null mammary tumors (Nguyen, Oketch-Rabah, et al. 2011). We conclude that the contribution of host microenvironment to cancer risk in mice following exposure to LDIR strongly depends on host genetic backgrounds.
Our studies demonstrate that genetic variations, likely acting both systemically and stromally, regulate the mammary microenvironment, which subsequently influences mammary cancer development. We identified 15 host genetic loci that control tumor latency, the majority of which (13 loci) were associated only with tumor latency after exposure to LDIR. This indicates a strong interaction between host genetics and LDIR in mammary tumor susceptibility. Surprisingly, 13 of 15 loci are also close to tumor susceptibility loci previously reported in other studies. The majority of these studies involved chemically-induced tumorigenesis (Mao & Balmain 2003; Demant 2003), suggesting common regulators of susceptibility between different types of environmental exposures. More interesting is the fact that the genetic loci identified in our study control host microenvironment, indicating that stromal microenvironment could also play an important role in chemical carcinogens susceptibilities.

Radiation-induced activation of pathways that control release of inflammatory cytokines varies among mouse strains (Tartakovsky et al. 1993; Peled et al. 1995; Haran Ghera et al. 1997) and may contribute to genetic susceptibility to radiation induced leukemia in mice (Tartakovsky et al. 1993). Bioinformatics analysis shows that the genetic loci identified in this study are enriched for genes involved in regulating the immune response, including signaling pathways of natural killer cells and cytokines. Consistent with this, we observed significant differences in plasma cytokine levels between SPRET/EiJ and BALB/c mice in response to LDIR, and found that differences in plasma cytokine levels in F1Bx mice are controlled by genetic variation, some of which correspond to loci identified in this study as regulating tumor frequency and latency. Moreover, certain plasma cytokine levels correlate with mammary tumor development after exposure to LDIR. These results indicate that the mammary tumor susceptibility to LDIR, at least in part, is controlled through the regulation of cytokines, although additional studies are required to identify the direct causes and consequences of cytokine levels and their genetic regulation.

Analysis of the upstream regulators of candidate genes within these loci revealed the TGFβ pathway as a potential mediator of mammary tumor susceptibility to LDIR. A

number of studies have demonstrated that TGFβ1 plays a critical role in radiation-induced breast cancer risk (Barcellos-Hoff et al. 2005; Barcellos-Hoff 2011; Moses & Barcellos-Hoff 2011). It has been proposed that TGFβ1 acts as a tumor suppressor at early stages of tumor outgrowth, either by suppressing growth and/or inducing differentiation (Cui et al. 1996; Roberts & Wakefield 2003; Sporn et al. 1989; Wakefield et al. 1995; Wakefield 2002). In this study, we found that compared to BALB/c, SPRET/EiJ mice have significantly higher TGF-β1 levels. Importantly, we demonstrate that the reduced branching and invasion observed in parental SPRET/EiJ mammary glands and ex vivo hybrid organoids requires high TGFβ1 levels, consistent with previous reports that the use of TGFβ1 pathway inhibits invasion and branching in 3D stromal collagen gels (Nelson et al. 2006). This could also explain resistance of SPRET/EiJ strain to tumor development in general, and here to mammary cancer risk following LDIR.

In summary, we used a comprehensive systems biology approach to identify genetic variations that control mammary cancer susceptibility by altering host responses to LDIR. We show that these genetic variations actually prevent or retard the effect of radiation to promote malignancy through host microenvironmental changes and/or systemic effects, including control of cytokines such as TGFβ1 levels. We propose that human homologues of these genes could be used to establish susceptibility in individuals, and may identify preventive strategies to identify the populations that are at higher risk for cancer.

Chapter 6 – Conclusions and future directions

This final chapter will boil down the results and conclusions from the previous chapters while providing insights about future directions and experiments. First a quick digest of the dissertation and the corresponding aims will be summarized. Afterwards a discussion of the aims will follow along with a specific analysis and further outlook.

6.1 – Results Summary

The goal of this dissertation was to understand the interaction between the epithelium and the microenvironment during mammary gland development. To dissect and analyze this interaction, the project was first divided into three distinct components focused on understanding the dynamic reciprocity of the epithelium and its surrounding microenvironment. From this distinction, this dissertation can be parsed into three distinct aims: 1) First, the development of a three-dimensional culture system for mouse mammary epithelial cells. 2) Next, the identification of the role of matrix-metalloproteinases in mammary epithelial architecture and function. 3) Last, the involvement of TGF-beta in mammary development and carcinogenesis.

6.1.1 – Determination of a three-dimensional culture system

For Aim I, we developed a novel culture system to explore mammary gland developmental processes *ex vivo* using three-dimensional culture models. Using either a laminin-rich extracellular matrix or a collagen-1 matrix, we were able to mimic two distinct developmental processes observed *in vivo* during mammary gland development. Utilizing this unique culture system sets the foundation for the subsequent work within this dissertation by emphasizing the utility of the three-dimensional cultures to recapitulate the complex milieu observed within the physiological environment.

In this aim, we sought to develop and produce a three-dimensional culture model to simulate the environment epithelial cells experience *in vivo*. Towards that end, we developed a method to extract epithelial mammary gland fragments in a process to preserve their capacity to undergo a morphological change when place either in a laminin-rich extracellular matrix or a collagen-1 gel. From there we were able to craft our experiment around this model. Further characterization of the model was demonstrated by visual observation via light microscopy and fluorescent imaging.

Here it was demonstrated that we are able to recapitulate two distinct mammary gland developmental processes through the variation of the culture matrix and medium. By altering the matrix by which the isolated epithelial fragments are maintained within, we can distinctive characterize the delicate process of either branching and elongation of the mammary epithelium or the development of the alveolar buds. It is remarkable, given that by varying the environment just slightly causes such a dramatic physiological change given that the epithelial cells are identical. Future iterations would involve developing a culture model that could possibly utilize a chemically defined matrix of synthesized and purified peptides to help elucidate more the factors involved in providing the signaling cues for the tissue.

6.1.2 – Involvement of matrix metalloproteinase 14 and branching morphogenesis

In Aim II, we utilized our developed three-dimensional model to explore the microenvironment and the function of matrix metalloproteinases in mammary gland specific function and architecture during development. First matrix metalloproteinase 14 was studied with the respect to its functional expression within the mammary gland during development and its spatial location. It was found that matrix metalloproteinase 14 was highly expressed during the elongation stage of the mammary gland and that it was located at the invading front of the mammary epithelial branches. Next, using three-dimensional cultures, we investigated further the relationship of matrix metalloproteinase 14 and the extracellular matrix. Focusing our studies on the collagen-1 matrix, we identified the collagen density controlling matrix metalloproteinase 14 dependent branching and the subsequent downstream signaling elements.

In addition, we presented an interesting new finding indicating that the transmembrane and/or cytoplasmic domain of matrix metalloproteinase 14 and not its catalytic domain functions in ERK activation and branching of the mammary gland fragments or mammary epithelial cell lines in a collagen-1 gel. We were able to provide evidence for a direct interaction between integrin beta-1 and matrix metalloproteinase 14, as well as a mutual regulation of integrin beta-1, matrix metalloproteinase 14, and ERK to conclude that these interactions regulate branching in the developing mammary epithelium. These findings concerning the non-proteolytic functions of matrix metalloproteinase 14 and the cross-regulation between integrins and other signaling systems have been studied in our labs and others but this work provides an extension of the previous findings to illustrate novel signaling mechanisms. Also useful are the findings that the physiological expression of matrix metalloproteinase 14 in the epithelium and not just the stromal tissues are functionally important in addition to their location of expression.

Various criticisms could be made from the resulting work, some with regards to the model system actually illustrating and mimicking the processes since *in vivo*. One could make the argument that branching morphogenesis or branching itself using our three-dimensional culture model simulates invasion rather than branches since the branched structures *in vivo* contain a lumen, a hollowed out space to allow milk to flow through. Other criticisms could revolve around the statements indicating matrix metalloproteinase 14 as a central entity that transduces signals from the extracellular matrix. Although these criticisms may yield some merit, both can be easily tested to be proven in favor of what I have stated in the previous chapter.

6.1.3 – TGF-β1 involvement in tumor susceptibility

Finally, Aim III, we aimed to address the interaction of the microenvironment involvement in a complex disease, such as cancer. Utilizing mammary carcinogenesis model to investigate the determinants for factors underlying the affect of the extracellular matrix on tumor formation, we initiated a study using BALB/c and SPRET/Ei mice. These mice

have previously been utilized in various tumorigenic studies to identify the signaling networks for cancer susceptibility. By leveraging this model and combining it with our three-dimensional system, we can systematically uncover unique extracellular matrix components responsible for tumor formation and cancer susceptibility.

What we discovered using both our mouse and culture model was a novel understanding of how the extracellular matrix behaves and its relationship with the epithelium. What we determined was that the genetic background is an extremely important factor in determining the risk of cancer susceptibility using low dose ionizing radiation exposure as a medium. We illustrated that the endogenous microenvironment is critical for regulating cancer progression using both the mouse and *in vitro* culture model and dissected out particular factors modulating cancer susceptibility.

Our initial studies of mammary carcinogenesis using this model revealed that p53 null organoid fragments increased the incidence and accelerated tumor formation in response to low dose radiation (Nguyen, Oketch-Rabah, et al. 2011). Regarding our study, we used the model and brought it a step further to understand the microenvironmental effects. Using a backcross model, we developed over 300 genetically unique strains of mice and identified how the genetic variations controlled cancer susceptibility. We identified several cytokines affecting cancer progression using genetic loci and verified them using protein arrays. One of the cytokines that was identified was TGF-β1.

Next to further confirm the role of TGF-β1 as a culprit in modulating cancer susceptibility we identified serum levels of TGF-β1 and correlated tumor progression based on those levels making the conclusion that higher levels of TGF-β1 expressed by the host microenvironment led to a increase in tumor latency. To dissect this phenomenon further, we applied our three-dimensional culture model using the SPRET/Ei organoids as surrogates to identify TGF-β1 a substantial mediator for growth and proliferation. Taken together, these results infer that TGF-β1 is a key mediator for tumor latency and that *in vitro* TGF-β1 is the key mediator for branching morphogenesis both provide sufficient evidence about the critical interaction between the microenvironment and cancer.

Various criticisms can be made from this work, one comment could relate to the usage of ionizing radiation as a surrogate for carcinogenesis or another about the mechanism by which mammary tumor susceptibility is due to and downstream signaling of TGF-β1. While ionizing radiation may not be a widely used method for initiating carcinogenesis versus various other methods, but what we do know about ionizing radiation is that it affects the mammary gland quite significantly providing us the perfect damaging agent to study mammary carcinogenesis.

6.2 – Conclusions and future directions

In characterizing the interaction between the microenvironment and the mammary epithelium, it is necessary to define specific components and the context to investigate particular interactions that act between the extracellular matrix and the epithelium. In the breast epithelium, there exists a basement membrane that consists of proteins such as laminin, collagen, and others that play a crucial role in the establishment of epithelial architecture. Despite our tremendous knowledge about the about the mammary epithelium, it's complex milieu of signals are still not well understood, nor are the targets of signaling pathways for dysregulation in malignancy. As such, we have pioneered the usage of three-dimensional culture models to act as surrogates for interactions seen within the mammary gland. By studying the cell-cell and cell-microenvironment network, we can observe and determine how mammary epithelial cells maintain their architecture and organization of which are frequently deregulated in cancer. The results described in the previous chapters outline the usage of a three-dimensional culture model as a surrogate system to dissect and analyze the interactions between the cell and the extracellular matrix. Using this system, we discover the role of matrix metalloproteinase 14 in mammary gland development and a unique feature demonstrated by the protein with its ability to control cell migration through the non-catalytic domain. Lastly, we identify particular gene networks within the basement membrane coordinating tumor progression and malignancy. Using this intricate *ex vivo* culture system and combining our current understanding of mammary gland biology, we are able to help understand and explain how the epithelium normally develops and becomes deregulated in malignancy.

There is still much to be done in the near future to progress our current understanding of mammary gland biology. While the initial steps of providing a model system is completed, there is still much to accomplish with regards to applying it to further to increase our understanding on how matrix metalloproteinase 14 interacts with integrins or study other developmental stages outside of branching and elongation, such as alveolargenesis. While initial work provides a foundation on the characteristics of how the mammary epithelium behaves during development, there is still a vast unknown of the step-by-step process by which the epithelium senses the microenvironment and reacts thus leading to the microenvironment to further reactive in response generating a dynamic reciprocity of responses between one another.

References

Alford, D. et al., 1998. Integrin-matrix interactions affect the form of the structures developing from human mammary epithelial cells in collagen or fibrin gels. *Journal of cell science*, 111 (Pt 4)(4), pp.521–532.

Annes, J.P., Munger, J.S. & Rifkin, D.B., 2003. Making sense of latent TGFbeta activation. *Journal of cell science*, 116(Pt 2), pp.217–224.

Ayad, S. et al., 1998. *The Extracellular Matrix Factsbook*, Academic Press.

Baciu, P.C., Suleiman, E.A. & Deryugina, E.I., 2003. Membrane type-1 matrix metalloproteinase (MT1-MMP) processing of pro-α< sub> v</sub> integrin regulates cross-talk between α< sub> v</sub> β< sub> 3</sub> …. *Experimental cell ….*

Balmain, A., 2002. Cancer as a Complex Genetic Trait. *Cell*, 108(2), pp.145–152.

Barcellos-Hoff, M.H., 2011. TGFβ Biology in Breast: 15 Years On. *Journal of mammary gland biology and neoplasia*, 16(2), pp.65–66.

Barcellos-Hoff, M.H. & Ravani, S.A., 2000. Irradiated mammary gland stroma promotes the expression of tumorigenic potential by unirradiated epithelial cells. *Cancer research*, 60(5), pp.1254–1260.

Barcellos-Hoff, M.H. et al., 1989. Functional differentiation and alveolar morphogenesis of primary mammary cultures on reconstituted basement membrane. *Development (Cambridge, England)*, 105(2), pp.223–235.

Barcellos-Hoff, M.H., Park, C. & Wright, E.G., 2005. Radiation and the microenvironment – tumorigenesis and therapy. *Nature reviews. Cancer*, 5(11), pp.867–875.

Bella, J. et al., 1994. Crystal and molecular structure of a collagen-like peptide at 1.9 A resolution. *Science (New York, N.Y.)*, 266(5182), pp.75–81.

Berdichevsky, F. et al., 1994. Branching morphogenesis of human mammary epithelial cells in collagen gels. *Journal of cell science*, 107 (Pt 12)(12), pp.3557–3568.

Bissell, D.M., 1981. Primary hepatocyte culture: substratum requirements and production of matrix components. *Federation proceedings*, 40(10), pp.2469–2473.

Bissell, M.J., 1981. The differentiated state of normal and malignant cells or how to define a “normal” cell in culture. *International review of cytology*, 70, pp.27–100.

Bissell, M.J. & Hines, W.C., 2011. Why don't we get more cancer? A proposed role of the microenvironment in restraining cancer progression. *Nature medicine*, 17(3), pp.320–329.

Bissell, M.J. & Radisky, D., 2001. Putting tumours in context. *Nature reviews. Cancer*, 1(1), pp.46–54.

Bittner, J.J., Evans, C.A. & Green, R.G., 1945. SURVIVAL OF THE MAMMARY TUMOR MILK AGENTS OF MICE. *Science (New York, N.Y.)*, 101(2613), pp.95–97.

Blackburn, A.C. et al., 2003. BALB/c alleles for Prkdc and Cdkn2a interact to modify tumor susceptibility in Trp53+/- mice. *Cancer research*, 63(10), pp.2364–2368.

Blackburn, D.G., 1993. Lactation: historical patterns and potential for manipulation. *Journal of dairy science*, 76(10), pp.3195–3212.

Bocchinfuso, W.P. et al., 2013. Induction of Mammary Gland Development in Estrogen Receptor-α Knockout Mice. *dx.doi.org*, 141(8), pp.2982–2994.

Boyd, N.F., 2007. Mammographic Density and the Risk and Detection of Breast Cancer — NEJM. *The New England journal of medicine*.

Boyd, N.F. et al., 1998. Mammographic densities and breast cancer risk. *Cancer Epidemiology Biomarkers & Prevention*, 7(12), pp.1133–1144.

Brownfield, D.G. et al., 2013. Patterned Collagen Fibers Orient Branching Mammary Epithelium through Distinct Signaling Modules. *Current Biology*, 23(8), pp.703–709.

Burgess, A. et al., 2010. Loss of human Greatwall results in G2 arrest and multiple mitotic defects due to deregulation of the cyclin B-Cdc2/PP2A balance. *Proceedings of the National Academy of Sciences*, 107(28), pp.12564–12569.

Calalb, M.B., Polte, T.R. & Hanks, S.K., 1995. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Molecular and cellular biology*, 15(2), pp.954–963.

Callahan, R. & Smith, G.H., 2000. MMTV-induced mammary tumorigenesis: gene discovery, progression to malignancy and cellular pathways. *Oncogene*, 19(8), pp.992–1001.

Cao, J. et al., 2004. Distinct roles for the catalytic and hemopexin domains of membrane type 1-matrix metalloproteinase in substrate degradation and cell migration. *The Journal of biological chemistry*, 279(14), pp.14129–14139.

Chen, W.Y. & Abatangelo, G., 1999. Functions of hyaluronan in wound repair. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society*, 7(2), pp.79–89.

Chun, T.-H. et al., 2010. Genetic link between obesity and MMP14-dependent adipogenic collagen turnover. *Diabetes*, 59(10), pp.2484–2494.

Cui, W. et al., 1996. TGFβ1 Inhibits the Formation of Benign Skin Tumors, but Enhances Progression to Invasive Spindle Carcinomas in Transgenic Mice. *Cell*, 86(4), pp.531–542.

Daniel, C. & Strickland, P., 1995. ScienceDirect.com - Developmental Biology - Expression and Functional Role of E- and P-Cadherins in Mouse Mammary Ductal Morphogenesis and Growth. *Developmental biology*.

Demant, P., 2003. Cancer susceptibility in the mouse: genetics, biology and implications for human cancer. *Nature reviews. Genetics*, 4(9), pp.721–734.

Donjacour, A.A. & Cunha, G.R., 1991. Stromal regulation of epithelial function. In M. E. Lippman & R. B. Dickson, eds. *Regulatory Mechanisms in Breast Cancer*. Cancer Treatment and Research. Boston, MA: Springer US, pp. 335–364.

Dudley, D.T. et al., 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proceedings of the National Academy of Sciences of the United States of America*, 92(17), pp.7686–7689.

E, J. et al., 2002. Oncogenic Ras/Her-2 mediate hyperproliferation of polarized epithelial cells in 3D cultures and rapid tumor growth via the PI3K pathway. *Oncogene*, 21(33), pp.5148–5159.

Emerman, J.T. & Pitelka, D.R., 1977. Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. *In vitro*, 13(5), pp.316–328.

Fata, J.E. et al., 2007. The MAPK(ERK-1,2) pathway integrates distinct and antagonistic signals from TGFalpha and FGF7 in morphogenesis of mouse mammary epithelium. *Developmental biology*, 306(1), pp.193–207.

Fata, J.E. et al., 1999. Timp-1 Is Important for Epithelial Proliferation and Branching Morphogenesis during Mouse Mammary Development. *Developmental ….*

Fata, J.E., Werb, Z. & Bissell, M.J., 2004. Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. *Breast cancer research : BCR*, 6(1), pp.1–11.

Friedl, P., 2004. Prespecification and plasticity: shifting mechanisms of cell migration. *Current opinion in cell biology*.

Fukuda, Y. et al., 1988. The role of interstitial collagens in cleft formation of mouse embryonic submandibular gland during initial branching. *Development (Cambridge, England)*, 103(2), pp.259–267.

Gilles, C. et al., 1997. Implication of collagen type I-induced membrane-type 1-matrix

metalloproteinase expression and matrix metalloproteinase-2 activation in the metastatic progression of breast carcinoma. *Laboratory investigation; a journal of technical methods and pathology*, 76(5), pp.651–660.

Gingras, D. et al., 2001. Activation of the extracellular signal-regulated protein kinase (ERK) cascade by membrane-type-1 matrix metalloproteinase (MT1-MMP). *FEBS letters*.

Gross, J. & Lapiere, C.M., 1962. Collagenolytic activity in amphibian tissues: a tissue culture assay. *Proceedings of the National Academy of Sciences of the United States of America*, 48(6), pp.1014–1022.

Gudjonsson, T. et al., 2002. Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. *Genes & development*, 16(6), pp.693–706.

Ha, H.Y. et al., 2001. Overexpression of membrane-type matrix metalloproteinase-1 gene induces mammary gland abnormalities and adenocarcinoma in transgenic mice. *Cancer research*, 61(3), pp.984–990.

Hanke, J.H. et al., 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *The Journal of biological chemistry*, 271(2), pp.695–701.

Haran Ghera, N. et al., 1997. Increased Circulating Colony-Stimulating Factor-1 (CSF-1) in SJL/J Mice With Radiation-Induced Acute Myeloid Leukemia (AML) Is Associated With Autocrine Regulation of AML Cells by CSF-1. *Blood*, 89(7), pp.2537–2545.

Hennighausen, L. & Robinson, G.W., 2005. Information networks in the mammary gland. *Nature reviews. Molecular cell biology*, 6(9), pp.715–725.

Hirai, Y. et al., 1998. Epimorphin functions as a key morphoregulator for mammary epithelial cells. *The Journal of cell biology*, 140(1), pp.159–169.

Hogg, N.A., Harrison, C.J. & Tickle, C., 1983. Lumen formation in the developing mouse mammary gland. *Journal of embryology and experimental morphology*, 73, pp.39–57.

Holmbeck, K. et al., 1999. MT1-MMP-deficient mice develop dwarfism, osteopenia, arthritis, and connective tissue disease due to inadequate collagen turnover. *Cell*.

Hotary, K.B. et al., 2002. Matrix metalloproteinases (MMPs) regulate fibrin-invasive activity via MT1-MMP-dependent and -independent processes. *The Journal of Experimental Medicine*, 195(3), pp.295–308.

Huhtala, P. et al., 1995. Cooperative signaling by alpha 5 beta 1 and alpha 4 beta 1 integrins regulates metalloproteinase gene expression in fibroblasts adhering to fibronectin. *The Journal of cell biology*, 129(3), pp.867–879.

Hynes, R.O., 2002. Integrins: bidirectional, allosteric signaling machines. *Cell*.

Ingman, W.V. et al., 2006. Macrophages promote collagen fibrillogenesis around terminal end buds of the developing mammary gland. *Developmental Dynamics*, 235(12), pp.3222–3229.

Itoh, Y. et al., 2001. Homophilic complex formation of MT1-MMP facilitates proMMP-2 activation on the cell surface and promotes tumor cell invasion. *The EMBO journal*, 20(17), pp.4782–4793.

Kanwar, Y.S. et al., 1999. Role of membrane-type matrix metalloproteinase 1 (MT-1-MMP), MMP-2, and its inhibitor in nephrogenesis. *American Journal of …*.

Keely, P.J., Wu, J.E. & Santoro, S.A., 1995. The spatial and temporal expression of the α2β1 integrin and its ligands, collagen I, collagen IV, and laminin, suggest important roles in mouse mammary morphogenesis. *Differentiation; research in biological diversity*, 59(1), pp.1–13.

Kessenbrock, K., Plaks, V. & Werb, Z., 2010. Matrix metalloproteinases: regulators of the tumor microenvironment. *Cell*, 141(1), pp.52–67.

Khoshnoodi, J., Pedchenko, V. & Hudson, B.G., 2008. Mammalian collagen IV. *Microscopy research and technique*, 71(5), pp.357–370.

Kim, H., Laing, M. & Muller, W., 2005. c-Src-null mice exhibit defects in normal mammary gland development and ERl[alpha]l signaling. *Oncogene*, 24(36), pp.5629–5636.

Klinghoffer, R.A. et al., 1999. Src family kinases are required for integrin but not PDGFR signal transduction. *The EMBO journal*, 18(9), pp.2459–2471.

Klinowska, T.C.M. et al., 1999. Laminin and β1 integrins are crucial for normal mammary gland development in the mouse. *Developmental …*.

Kuslak, S.L. & Marker, P.C., 2007. Fibroblast growth factor receptor signaling through MEK–ERK is required for prostate bud induction. *Differentiation; research in biological diversity*, 75(7), pp.638–651.

Langholz, O. et al., 1995. Collagen and collagenase gene expression in three-dimensional collagen lattices are differentially regulated by alpha 1 beta 1 and alpha 2 beta 1 integrins. *The Journal of cell biology*, 131(6 Pt 2), pp.1903–1915.

Lee, P.P.H. et al., 2001. Functional role of matrix metalloproteinases (MMPs) in

mammary epithelial cell development. *Journal of cellular physiology*, 188(1), pp.75–88.

Liu, H. et al., 2004. Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. *The Journal of cell biology*, 164(4), pp.603–612.

Lo, A.T. et al., 2012. Constructing Three-Dimensional Models to Study Mammary Gland Branching Morphogenesis and Functional Differentiation. *Journal of mammary gland biology and neoplasia*.

Mao, J.-H. & Balmain, A., 2003. Genomic approaches to identification of tumour-susceptibility genes using mouse models. *Current opinion in genetics & development*, 13(1), pp.14–19.

Maru, Y. et al., 1998. Virally activated ras cooperates with integrin to induce tubulogenesis in sinusoidal endothelial cell lines. *Journal of cellular physiology*, 176(2), pp.223–234.

Massagué, J., 2012. TGFβ signalling in context. *Nature reviews. Molecular cell biology*, 13(10), pp.616–630.

Matthew D Shoulders, R.T.R., 2009. COLLAGEN STRUCTURE AND STABILITY. *Annual review of biochemistry*, 78(1), pp.929–958.

Maxwell, C.A. et al., 2008. Targeted and nontargeted effects of ionizing radiation that impact genomic instability. *Cancer research*, 68(20), pp.8304–8311.

Medina, D., 1996. The mammary gland: a unique organ for the study of development and tumorigenesis. *Journal of mammary gland biology and neoplasia*, 1(1), pp.5–19.

Meyer, T.N. et al., 2004. Spatiotemporal regulation of morphogenetic molecules during in vitro branching of the isolated ureteric bud: toward a model of branching through budding in the developing kidney. *Developmental biology*, 275(1), pp.44–67.

Michalopoulos, G. & Pitot, H.C., 1975. Primary culture of parenchymal liver cells on collagen membranes. Morphological and biochemical observations. *Experimental cell research*, 94(1), pp.70–78.

Michalopoulos, G. et al., 1976. Cytochrome P-450 induction by phenobarbital and 3-methylcholanthrene in primary cultures of hepatocytes. *Science (New York, N.Y.)*, 193(4256), pp.907–909.

Mienaltowski, M.J. & Birk, D.E., 2014. Structure, physiology, and biochemistry of collagens. *Advances in experimental medicine and biology*, 802(Chapter 2), pp.5–29.

Miner, J.H. & Yurchenco, P.D., 2004. LAMININ FUNCTIONS IN TISSUE MORPHOGENESIS. *dx.doi.org*, 20(1), pp.255–284.

Montesano, R., Schaller, G. & Orci, L., 1991. Induction of epithelial tubular morphogenesis in vitro by fibroblast-derived soluble factors. *Cell*, 66(4), pp.697–711.

Moore, K.A. et al., 2005. Control of basement membrane remodeling and epithelial branching morphogenesis in embryonic lung by Rho and cytoskeletal tension. *Developmental Dynamics*, 232(2), pp.268–281.

Mori, H. et al., 2002. CD44 directs membrane-type 1 matrix metalloproteinase to lamellipodia by associating with its hemopexin-like domain. *The EMBO journal*, 21(15), pp.3949–3959.

Mori, H. et al., 2009. Self-organization of engineered epithelial tubules by differential cellular motility. *Proceedings of the National Academy of Sciences*, 106(35), pp.14890–14895.

Mori, H. et al., 2013. Transmembrane/cytoplasmic, rather than catalytic, domains of Mmp14 signal to MAPK activation and mammary branching morphogenesis via binding to integrin β1. *Development (Cambridge, England)*, 140(2), pp.343–352.

Moses, H. & Barcellos-Hoff, M.H., 2011. TGF-β Biology in Mammary Development and Breast Cancer. *Cold Spring Harbor perspectives in biology*, 3(1), pp.a003277–a003277.

Muraoka, R.S. et al., 2003. Increased malignancy of Neu-induced mammary tumors overexpressing active transforming growth factor beta1. *Molecular and cellular biology*, 23(23), pp.8691–8703.

Myers, C.A. et al., 1998. Characterization of BCE-1, a transcriptional enhancer regulated by prolactin and extracellular matrix and modulated by the state of histone acetylation. *Molecular and cellular biology*, 18(4), pp.2184–2195.

Naylor, M.J. & Ormandy, C.J., 2002. Mouse strain-specific patterns of mammary epithelial ductal side branching are elicited by stromal factors. *Developmental dynamics : an official publication of the American Association of Anatomists*, 225(1), pp.100–105.

Nelson, C.M. et al., 2006. Tissue geometry determines sites of mammary branching morphogenesis in organotypic cultures. *Science (New York, N.Y.)*, 314(5797), pp.298–300.

Neville, M.C. & Daniel, C.W., 1987. *The Mammary gland*, Plenum Publishing Corporation.

Nguyen, D.H., Martinez-Ruiz, H. & Barcellos-Hoff, M.H., 2011. Consequences of epithelial or stromal TGFβ1 depletion in the mammary gland. *Journal of mammary gland biology and neoplasia*, 16(2), pp.147–155.

Nguyen, D.H., Oketch-Rabah, H.A., et al., 2011. Radiation acts on the microenvironment to affect breast carcinogenesis by distinct mechanisms that decrease cancer latency and affect tumor type. *Cancer cell*, 19(5), pp.640–651.

Nonaka, T. et al., 2005. Competitive disruption of the tumor-promoting function of membrane type 1 matrix metalloproteinase/matrix metalloproteinase-14 in vivo. *Molecular cancer therapeutics*, 4(8), pp.1157–1166.

Novaro, V., Roskelley, C.D. & Bissell, M.J., 2003. Collagen-IV and laminin-1 regulate estrogen receptor alpha expression and function in mouse mammary epithelial cells. *Journal of cell science*, 116(Pt 14), pp.2975–2986.

O'Brien, L.E. et al., 2004. ERK and MMPs sequentially regulate distinct stages of epithelial tubule development. *Developmental cell*.

Ohuchi, E. et al., 1997. Membrane type 1 matrix metalloproteinase digests interstitial collagens and other extracellular matrix macromolecules. *The Journal of biological chemistry*, 272(4), pp.2446–2451.

Okayasu, R. et al., 2000. A deficiency in DNA repair and DNA-PKcs expression in the radiosensitive BALB/c mouse. *Cancer research*, 60(16), pp.4342–4345.

Page-McCaw, A., Ewald, A.J. & Werb, Z., 2007. Matrix metalloproteinases and the regulation of tissue remodelling. *Nature reviews. Molecular cell biology*, 8(3), pp.221–233.

Paszek, M.J. et al., 2005. Tensional homeostasis and the malignant phenotype. *Cancer cell*, 8(3), pp.241–254.

Peled, A., Tzehoval, E. & Haran-Ghera, N., 1995. Role of cytokines in termination of the B cell lymphoma dormant state in AKR mice. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K*, 9(6), pp.1095–1101.

Pérez-Losada, J., Castellanos-Martín, A. & Mao, J.-H., 2011. Cancer evolution and individual susceptibility. *Integrative biology : quantitative biosciences from nano to macro*, 3(4), pp.316–328.

Pinkas, J. & Leder, P., 2002. MEK1 signaling mediates transformation and metastasis of EpH4 mammary epithelial cells independent of an epithelial to mesenchymal transition. *Cancer research*, 62(16), pp.4781–4790.

Provenzano, P.P. et al., 2009. Matrix density-induced mechanoregulation of breast cell

phenotype, signaling and gene expression through a FAKl[ndash]lERK linkage. *Oncogene*, 28(49), pp.4326–4343.

Radisky, D.C. et al., 2005. Rac1b and reactive oxygen species mediate MMP-3-induced EMT and genomic instability. *Nature*, 436(7047), pp.123–127.

Reichmann, E. et al., 1989. New mammary epithelial and fibroblastic cell clones in coculture form structures competent to differentiate functionally. *The Journal of cell biology*, 108(3), pp.1127–1138.

Rillema, J.A., 1994. Development of the mammary gland and lactation. *Trends in endocrinology and metabolism: TEM*, 5(4), pp.149–154.

Roberts, A.B. & Wakefield, L.M., 2003. The two faces of transforming growth factor beta in carcinogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, 100(15), pp.8621–8623.

Robinson, G.W., 2007. Cooperation of signalling pathways in embryonic mammary gland development. *Nature reviews. Genetics*, 8(12), pp.963–972.

Robinson, G.W., 2004. Identification of signaling pathways in early mammary gland development by mouse genetics. *Breast cancer research : BCR*, 6(3), pp.105–108.

Rousseau, J.C. et al., 1996. Processing of type XI collagen. Determination of the matrix forms of the alpha1(XI) chain. *The Journal of biological chemistry*, 271(39), pp.23743–23748.

Rozanov, D.V. et al., 2001. Mutation analysis of membrane type-1 matrix metalloproteinase (MT1-MMP). The role of the cytoplasmic tail Cys(574), the active site Glu(240), and furin cleavage motifs in oligomerization, processing, and self-proteolysis of MT1-MMP expressed in breast carcinoma cells. *The Journal of biological chemistry*, 276(28), pp.25705–25714.

Rudolph-Owen, L.A. et al., 1998. The matrix metalloproteinase matrilysin influences early-stage mammary tumorigenesis. *Cancer research*, 58(23), pp.5500–5506.

Sabeh, F., Li, X.-Y., et al., 2009. Secreted versus membrane-anchored collagenases: relative roles in fibroblast-dependent collagenolysis and invasion. *Journal of Biological Chemistry*, 284(34), pp.23001–23011.

Sabeh, F., Shimizu-Hirota, R. & Weiss, S.J., 2009. Protease-dependent versus -independent cancer cell invasion programs: three-dimensional amoeboid movement revisited. *The Journal of cell biology*, 185(1), pp.11–19.

Samuels, M.L. et al., 1993. Conditional transformation of cells and rapid activation of the mitogen-activated protein kinase cascade by an estradiol-dependent human raf-1

protein kinase. *Molecular and cellular biology*, 13(10), pp.6241–6252.

Schedin, P. & Keely, P.J., 2011. Mammary gland ECM remodeling, stiffness, and mechanosignaling in normal development and tumor progression. *Cold Spring Harbor perspectives in biology*, 3(1), pp.a003228–a003228.

Sekhri, K.K., Pitelka, D.R. & DeOme, K.B., 1967. Studies of mouse mammary glands. I. Cytomorphology of the normal mammary gland. *Journal of the National Cancer Institute*, 39(3), pp.459–490.

Silberstein, G.B. et al., 1990. Epithelium-dependent extracellular matrix synthesis in transforming growth factor-beta 1-growth-inhibited mouse mammary gland. *The Journal of cell biology*, 110(6), pp.2209–2219.

Simian, M. et al., 2001. The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. *Development (Cambridge, England)*, 128(16), pp.3117–3131.

Slorach, E.M. & Werb, Z., 2003. Epithelial Morphogenesis: Netrin Comes to a Sticky and Terminal End. *Current Biology*, 13(12), pp.R491–R493.

Sonnenberg, A. et al., 1986. Development of mouse mammary gland: identification of stages in differentiation of luminal and myoepithelial cells using monoclonal antibodies and polyvalent antiserum against keratin. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 34(8), pp.1037–1046.

Spencer, V.A. et al., 2011. Depletion of nuclear actin is a key mediator of quiescence in epithelial cells. *Journal of cell science*, 124(Pt 1), pp.123–132.

Spooner, B.S. & Faubion, J.M., 1980. Collagen involvement in branching morphogenesis of embryonic lung and salivary gland. *Developmental biology*.

Sporn, M.B. et al., 1989. Transforming growth factor-beta and suppression of carcinogenesis. *Princess Takamatsu symposia*, 20, pp.259–266.

Srinivasan, K. et al., 2003. Netrin-1/neogenin interaction stabilizes multipotent progenitor cap cells during mammary gland morphogenesis. *Developmental cell*, 4(3), pp.371–382.

Sternlicht, M.D., 2006. Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. *Breast cancer research : BCR*, 8(1), p.201.

Sternlicht, M.D. et al., 1999. The stromal proteinase MMP3/stromelysin-1 promotes mammary carcinogenesis. *Cell*, 98(2), pp.137–146.

Sternlicht, M.D., Bissell, M.J. & Werb, Z., 2000. The matrix metalloproteinase stromelysin-1 acts as a natural mammary tumor promoter. *Oncogene*, 19(8), pp.1102–1113.

Streuli, C.H. et al., 1993. Extracellular matrix regulates expression of the TGF-beta 1 gene. *The Journal of cell biology*, 120(1), pp.253–260.

Streuli, C.H. et al., 1995. Laminin mediates tissue-specific gene expression in mammary epithelia. *The Journal of cell biology*, 129(3), pp.591–603.

Streuli, C.H., Bailey, N. & Bissell, M.J., 1991. Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. *The Journal of cell biology*, 115(5), pp.1383–1395.

Sympson, C.J. et al., 1994. Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *The Journal of cell biology*, 125(3), pp.681–693.

Szabova, L. et al., 2005. Expression pattern of four membrane-type matrix metalloproteinases in the normal and diseased mouse mammary gland. *Journal of cellular physiology*, 205(1), pp.123–132.

Takino, T. et al., 2004. Membrane type 1 matrix metalloproteinase regulates collagen-dependent mitogen-activated protein/extracellular signal-related kinase activation and cell migration. *Cancer research*, 64(3), pp.1044–1049.

Talhouk, R.S. et al., 1991. Proteinases of the mammary gland: developmental regulation in vivo and vectorial secretion in culture. *Development (Cambridge, England)*, 112(2), pp.439–449.

Tartakovsky, B. et al., 1993. Low doses of radiation induce systemic production of cytokines: Possible contribution to leukemogenesis. *International Journal of Cancer*, 55(2), pp.269–274.

Tomasek, J.J. et al., 1997. Gelatinase A Activation Is Regulated by the Organization of the Polymerized Actin Cytoskeleton. *The Journal of biological chemistry*, 272(11), pp.7482–7487.

Ueno, H. et al., 1997. Expression and tissue localization of membrane-types 1, 2, and 3 matrix metalloproteinases in human invasive breast carcinomas. *Cancer research*, 57(10), pp.2055–2060.

van Dijk, F.S. et al., 2011. Osteogenesis Imperfecta: A Review with Clinical Examples. *Molecular syndromology*, 2(1), pp.1–20.

Visse, R. & Nagase, H., 2003. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circulation research*, 92(8), pp.827–839.

Wagner, K.-U. et al., 2001. Spatial and temporal expression of the Cre gene under the control of the MMTV-LTR in different lines of transgenic mice. *Transgenic Research*, 10(6), pp.545–553.

Wakefield, L., 2002. TGF-Î² signaling: positive and negative effects on tumorigenesis. *Current opinion in genetics & development*, 12(1), pp.22–29.

Wakefield, L.M. et al., 1995. Transforming growth factor-beta s in mammary tumorigenesis: promoters or antipromoters? *Progress in clinical and biological research*, 391, pp.133–148.

Weaver, V.M. et al., 1997. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. *The Journal of cell biology*, 137(1), pp.231–245.

Weinberg, R., 2013. *The biology of cancer* 2nd ed., Garland Science.

White, D.E. et al., 2004. Targeted disruption of β1-integrin in a transgenic mouse model of human breast cancer reveals an essential role in mammary tumor induction. *Cancer cell*.

Williams, J.M. & Daniel, C.W., 1983. Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. *Developmental biology*, 97(2), pp.274–290.

Wiseman, B.S. et al., 2003. Site-specific inductive and inhibitory activities of MMP-2 and MMP-3 orchestrate mammary gland branching morphogenesis. *The Journal of cell biology*, 162(6), pp.1123–1133.

Witty, J.P., Wright, J.H. & Matrisian, L.M., 1995. Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. *Molecular biology of the cell*, 6(10), pp.1287–1303.

Wolf, K. et al., 2003. Compensation mechanism in tumor cell migration: mesenchymal-amoeboid transition after blocking of pericellular proteolysis. *The Journal of cell biology*, 160(2), pp.267–277.

Wolf, K. et al., 2007. Multi-step pericellular proteolysis controls the transition from individual to collective cancer cell invasion. *Nature cell biology*, 9(8), pp.893–904.

Xu, R. et al., 2009. Sustained activation of STAT5 is essential for chromatin remodeling

and maintenance of mammary-specific function. *The Journal of cell biology*, 184(1), pp.57–66.

Yana, I. et al., 2007. Crosstalk between neovessels and mural cells directs the site-specific expression of MT1-MMP to endothelial tip cells. *Journal of cell science*, 120(Pt 9), pp.1607–1614.

Yu, Y. et al., 2001. Elevated breast cancer risk in irradiated BALB/c mice associates with unique functional polymorphism of the Prkdc (DNA-dependent protein kinase catalytic subunit) gene. *Cancer research*, 61(5), pp.1820–1824.

Zhang, P.*, Lo, A.* et al., 2015. Identification of genetic loci that control mammary tumor susceptibility through the host microenvironment. *Scientific reports*, 5, p.8919. *(these authors contributed equally to this work)